Evaluation of herbs as potential alternatives for bear bile and rhino horn used in traditional Chinese medicines: chemical and biological analysis

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Abstract

There is currently an unsustainable rate of exploitation of certain species of plants and animals for use in traditional medicines. Bear bile and rhino horn are derived from endangered species and are traditionally combined with medicinal herbs as anti-bacterial and anti-inflammatory agents. This study was designed to assess herbs for use as possible alternatives for these two products. Herbs were selected based on their traditional use as anti-inflammatory and anti-bacterial agents. Chemical methods were used to confirm the plant species of the herbs and to measure concentrations of metals and pesticide residues as a means for assessing their quality. Anti-bacterial activities were determined using a direct bioautography technique. Anti-inflammatory activities of the herb extracts and isolated compounds were ascertained using an *in vitro* nuclear factor kappaB (NF-κB) activity, as assessed by IL-6 luciferase gene reporter assay. A novel assay was developed to estimate drug-herb interactions by measuring the effects of selected herbs and drugs on the production of eicosatrienoic acids from hepatic cytochrome P450 (CYP450) metabolism of arachidonic acid. In addition, CYP3A4 enzyme assays were conducted. The pesticide residue and heavy metal concentrations of the tested herbs was found not to exceed the existing legally permitted concentrations in foodstuffs, but the rhino horn sample contained elevated levels of Cd, Pb, Hg and Zn. Rhino horn was not found to be an effective anti-bacterial or anti-inflammatory agent in the assays used in this study. Seventeen herbs demonstrated anti-bacterial activity. Also, nine herbs demonstrated inhibitory NF-κB activity. Preliminary results indicate that co-administration of *Scutellaria baicalensis*, *Salvia miltiorrhiza*, *Rehmannia glutinosa* or Coptidis Rhizoma with drugs metabolised by CYP3A4, could lead to possible drug-herb interactions. Based on the information obtained in these studies nine herbs are proposed as alternatives to rhino horn; eight herbs and two Kampo medicines are proposed as alternatives to bear bile.

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CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1. Introduction

The use of traditional medicine (TM) containing ingredients obtained from animals and plants has maintained its popularity in all regions of the developing world and is gaining in popularity in industrialised countries (WHO, 2004; Easterford *et al.*, 2005). The popularity of TM has created a global market for herbal medicines that currently stands at over US $ 60 billion annually and is growing steadily (WHO, 2004).

Rhinoceroses (rhinos) and six species of bears are among animals and plants that are threatened with extinction and listed under Appendix I of the Convention on International Trade in Endangered Species (CITES) which bans international trade in their parts between CITES member states (CITES, 2006). However, there is continued use of rhino horn and bear parts in TM (Highley and Highley, 1994; Still, 2003). Signatories to CITES have expressed concern over the continued and uncontrolled use of several endangered species in traditional medicine in view of the potential threat to the long-term survival of these species and the need to develop traditional medicines on a sustainable basis (CITES, 1997).

1.2. Rhino Horn

The black rhino (*Diceros bicornis*, Rhinocerotidae) population decreased by 95% between 1970 and 1993 (WWF, 2002). Due to this population decline, all five surviving species (*Diceros bicornis*, *Cerathotherium sinum*, *Dicerorhinus sumatrensis*, *Rhinoceros sondaicus*, *Rhinoceroos unicornis*) of Rhinocerotidae are listed under CITES Appendix I (2006). However, populations of the one subspecies, *Cerathotherium sinum simum* in Africa and Swaiziland are included in Appendix II (CITES, 2006). Water buffalo horn has been used as an animal substitute for rhino horn, but generally at higher doses (Bensky and Barolet, 1990; Zou, 1989). Other alternatives to

rhino horn include horns from cattle and the saiga antelope (But *et al.*, 1990).

1.2.1. Rhino horn and its constituents

The primary constituent of rhino horn is keratin and other constituents include other proteins, amino acids, peptides, sterols, amines and calcium (Ingaki and Oida, 1970; Lee and Kim, 1974; Chang and But, 1987). Aqueous extracts of horns (12.5 g/kg) from water buffalo (*Bubalus bubalis*) and rhino have been reported to be antipyretic in rats (But *et al.*, 1990). Also, combined horn-herb extracts (containing approximately 2.5 g/kg of either rhino horn or buffalo horn) were found to be antipyretic (But and Tam, 1991). However, in another study, the rhino horn extract was not antipyretic in rabbits at an equivalent oral human dosage (0.05 g/kg) and a 10-fold human dosage (0.5 g/kg) (Laburn and Mitchel, 1997). But *et al.* (1990) also cited other studies conducted in Asia on the antipyretic properties of rhino horn with contradictory conclusions, but mainly with negative results. Generally, scientific research into the antipyretic properties of rhino horn has shown that it is effective in reducing temperature in febrile animals but only at high concentrations.

1.3. Bear Bile

In traditional Chinese medicine (TCM), bear bile was originally obtained from two members of the Ursidae family, namely, *Selenarctos thibetanus* (Asiatic black bear) and *Ursus arctos* (brown bear) (Chang and But, 1987; Bensky and Gamble, 1993). There is evidence to suggest that other species of bears such as *Ursus americanus* (American black bear) and *Helarctos malayanus* (Sun bear) have also been exploited (Lin *et al.*, 1997). *Selenarctos thibetanus (Ursus thibetanus)*, *Helarctos malayanus* and *Melursus ursinus* (sloth bear) and *Ursus arctos* (the populations of Bhutan, China, Mexico and Mongolia) are among the bear species listed under CITES Appendix I (2006). However, due to demand for bear products in TCM, regulated bear farming is in operation in China and the Republic of Korea,

where bile is artificially drained from bear gall bladders (Li, 2004). As a substitute to bear bile, bile derived from pig, water buffalo, goat, cattle and chicken have also been used in TCM and several of these have been sold as 'bear bile' (Lin *et al.*, 1997).

1.3.1. Use of bear bile constituents in Western medicine

Bear bile is composed of deconjugated tauroursodeoxycholic acid, taurochenodeoxycholic acid and taurocholic acid, of which the primary bile acids are known as ursodeoxycholic acid (UDCA), chenodeoxycholic acid and cholic acid, respectively (Espinoza *et al.*, 1993; Fig. 1.1). The bile acid pool of Asiatic black bears has been reported to contain over 50% UDCA (Espinoza *et al.*, 1993; Lin *et al.* 1997) compared to the trace levels found in humans (Kitani *et al.*, 1999; Kowdley, 2000). In the UK, pharmaceutical products containing UDCA (e.g. Destolit®, Urdox®, Ursofalk®, Ursogal®) are indicated for the dissolution of cholesterol gallstones (British Pharmacopoeia, 2000). UDCA has also been used in the treatment of some chronic inflammatory disorders such as liver fibrosis and chronic active hepatitis (Kowdley, 2000; Rolo *et al.*, 2000; Van Den Bogaert *et al.*, 2003). Also, UDCA has demonstrated anti-oxidant properties through the inhibition of reactive oxygen species and reduction of excessive accumulation of Ca^{2+} (Kitani *et al.*, 1999; Lee, 1999). In addition, UDCA has been reported to possess cardioprotective characteristics related, in part, to its anti-oxidant properties (Lee, 1999).

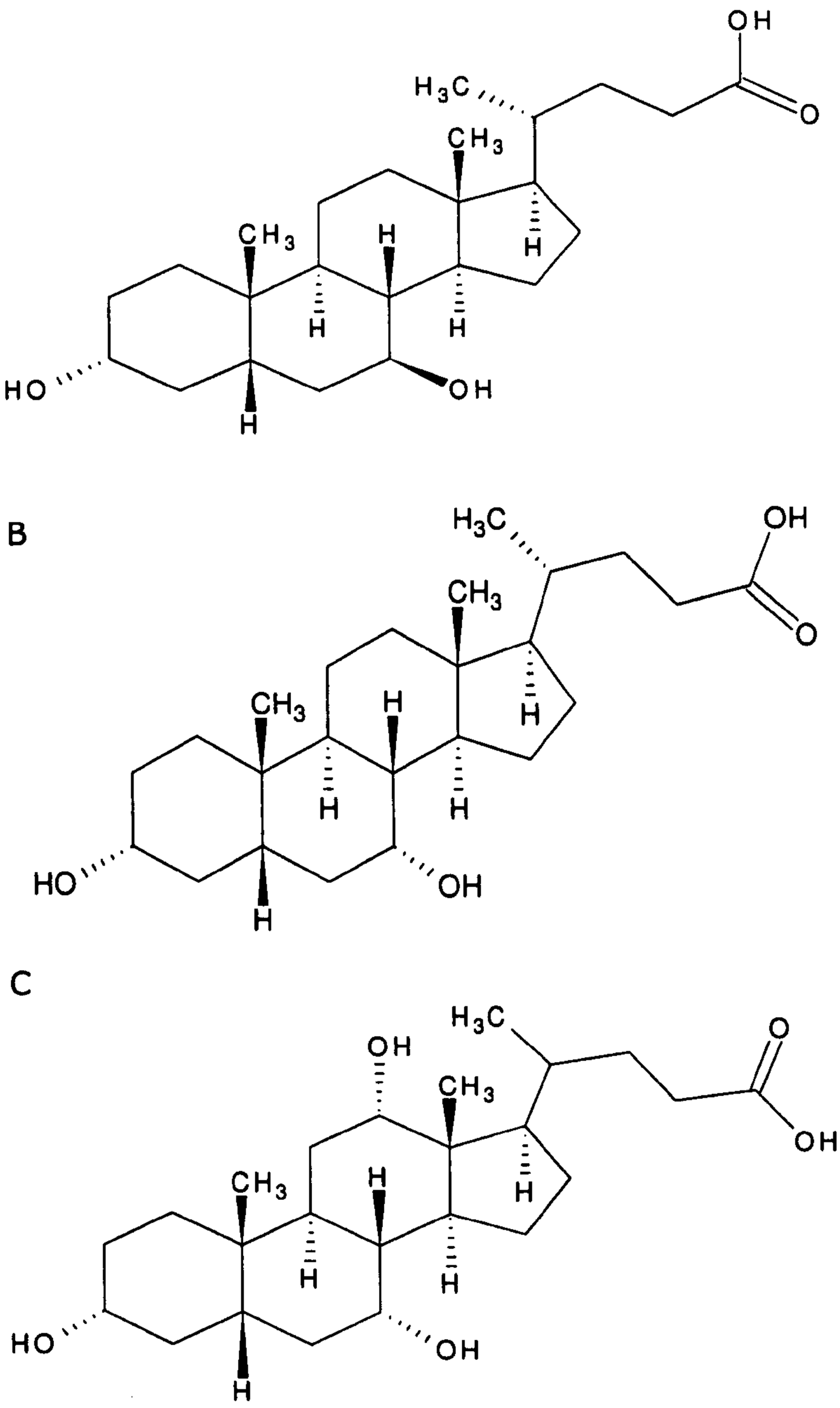

Fig. 1.1. The chemical structures of the primary bile acids found in bear bile.
(A) ursodeoxycholic acid (UDCA), 3α,7β-dihydroxy-5β-cholan-24-oic acid, ($C_{24}H_{40}O_4$)
(B) chenodeoxycholic acid (CDCA), 3α,7α-dihydroxy-5β-cholan-24-oic acid ($C_{24}H_{40}O_4$)
(C) cholic acid, 3α,7α,12α-trihydroxy-5β-cholanic acid, ($C_{24}H_{40}O_5$)

1.4. Objectives

The overall aim of this research was to provide scientific data to support the promotion of alternatives to the use of bear bile and rhino horn in TM and to help prevent the further depletion of threatened and endangered wildlife. The following objectives were used as a basis for the research.

To select appropriate herbs for investigation

To identify the plant species of the herbs using chemical analysis

To quantify metals in the herbs and rhino horn

To measure pesticide residues in the herbs

To evaluate potential pharmacological properties of the herbs and rhino horn using anti-bacterial tests

To test for possible mechanism of anti-inflammatory action

To assess potential drug-herb interactions

To propose herbs as alternatives to bear bile and rhino horn

1.5. Outline of Thesis

The thesis is divided into eight chapters. Following this overview, Chapter 2 presents the rationale for the selection of herbs, identifying both the herbs and the combinations of herbs (prescriptions) chosen. Chapter 3 describes the authentication method used to identify the plant species, as well as the metal and pesticide residue analysis. Chapter 4 describes the extraction and fractionation methods used to prepare the herbs for activity tests and chemical analysis. The anti-bacterial, nuclear factor kappaB and cytochrome P450 tests are detailed in Chapters 5, 6 and 7, respectively. Conclusions and suggested further research work are outlined and described in Chapter 8. A flowchart showing the different methods employed in the study and the collaborating institutions is shown in Fig. 1.2.

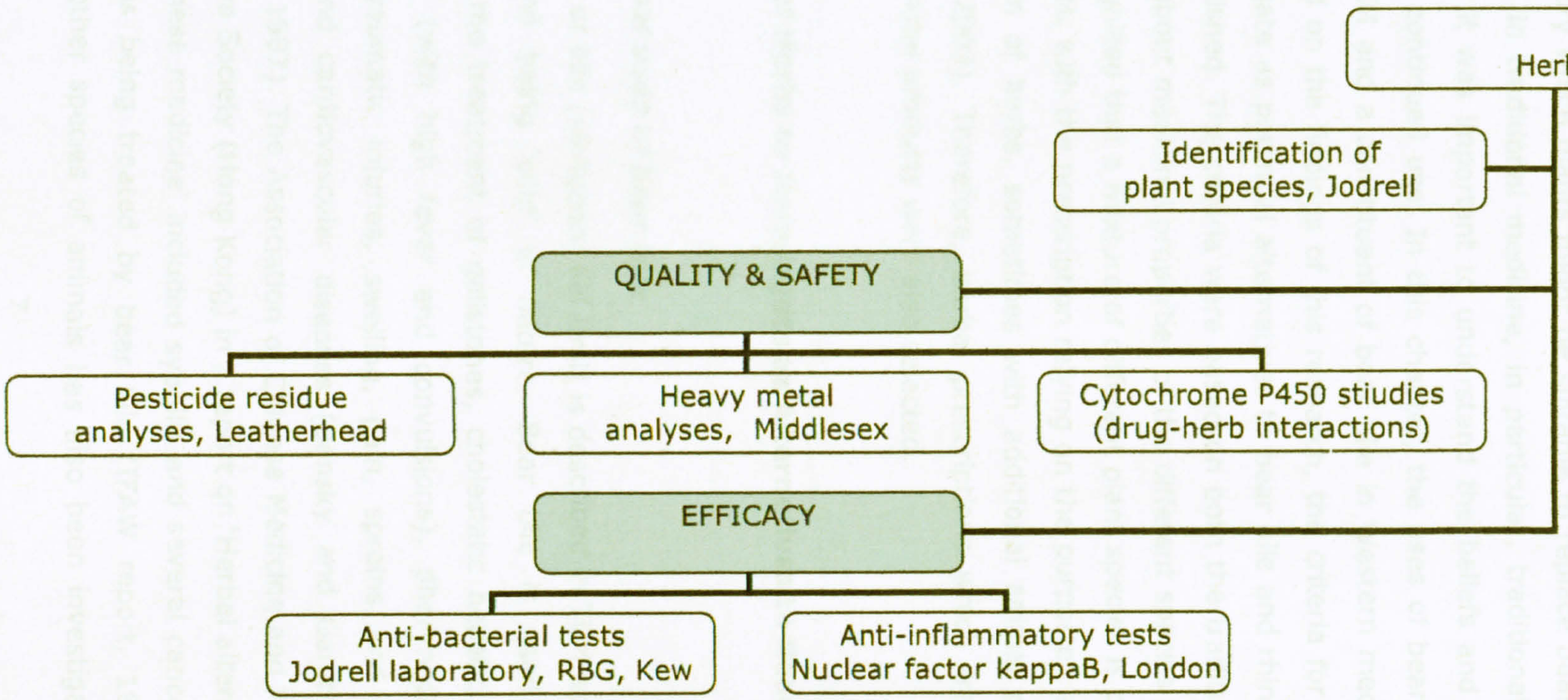

Fig. 1.2. The different methods employed in the study and the collaborating institutions.

CHAPTER 2. RATIONALE FOR SELECTION OF HERBS

2.1. Introduction

In order to identify which plants might be suitable to replace bear bile and rhino horn used in traditional medicine, in particular, traditional Chinese medicine (TCM), it was important to understand the beliefs and evidence supporting their continued use. In this chapter, the uses of bear bile and rhino horn in TCM and a constituent of bear bile in Western medicine are discussed. Based on the findings of this research, the criteria for choosing herbs to investigate as potential alternatives to bear bile and rhino horn in TCM were established. The criteria were based on both the traditional uses and knowledge about medicinal properties of the different species of plants. It was also recognised that a mixture of different plant species is commonly prescribed in TCM, with the prescription relying on the purported synergism of a combination of herbs, sometimes with additional animal parts and minerals (Still, 2003). Therefore, herbal prescriptions which traditionally contained the animal products were also selected.

2.2. Selection of Herbs to Investigate as Alternatives to Bear Bile

2.2.1. Traditional uses of bear bile

Bear gallbladder or bile (xiongdan; Fel Ursi) is described in TCM as having a 'bitter' taste and being 'cold' in nature. Bear bile is used by TCM practitioners in the treatment of gallstones, cholestatic hepatic diseases, febrile diseases (with high fever and convulsions), pharyngolaryngitis, conjunctivitis, traumatic injuries, swelling, pain, sprains and fractures, haemorrhoids and cardiovascular diseases (Bensky and Gamble, 1993; Chang and But, 1987). The Association of Chinese Medicine and Philosophy and the Earthcare Society (Hong Kong) in a report on 'Herbal alternatives to bear bile in Chinese medicine' included syphilis and several cancers in the list of conditions being treated by bear bile (IFAW report, 1994). Bile obtained from other species of animals has also been investigated. One

study showed bear bile and pig bile to demonstrate comparable anti-inflammatory, analgesic and anti-convulsant properties and therefore, pig bile has been advocated as a suitable animal alternative to bear bile (Li *et al.*, 1995).

2.2.2. Criteria used for selecting single herbs

There is scientific evidence to support the traditional use of bear bile in the treatment of inflammatory conditions (Li *et al.*, 1995) and its chief constituent, ursodeoxycholic acid (UDCA), in the treatment of cholesterol gallstones and chronic liver inflammation (Van Den Bogaert *et al.*, 2003), some cardiovascular diseases (Lee, 1999) and cancer (Im and Martinez, 2004). At the beginning of this study an extensive literature survey was conducted to select plant species used in medicinal herbal drugs that would be investigated as possible alternatives to bear bile. Sources of information included TCM Materia Medicas, pharmacology books and the Chinese and Japanese Pharmacopoeias. One hundred and three 'heat-clearing' herbs used in TCM were identified (Appendix I). Several of these medicinal herbs can be prepared from more than one plant species.

In addition to selecting herbs based on the TCM literature, species of plants with constituents similar in function and structure to UDCA (Fig. 1.1) were identified. For example, pentacyclic triterpenoids such as ursolic and oleanolic acid are attributed with anti-inflammatory, hepatoprotective and anti-neoplastic activities (Saraswat *et al.*, 2000; Syrovets *et al.*, 2000). However, these compounds are found in several plant species. Therefore, in consultation with TCM practitioners, the criteria for selecting the plant species for this study were further refined (Table 2.1). Priority was given to plant species that complied with the TCM properties of bear bile (Table 2.1; criteria (a) – (d)). All the herbs selected for this study are traditionally used in the practice of Chinese medicine. Additional criteria (Table 2.1; criteria; (e) – (h)) for choice were based on evidence from published scientific studies. The potential for using the selected herbs as alternatives to bear bile was discussed with TCM practitioners. These discussions resulted in a reduction in the number of individual herbs to be investigated from 103 (Appendix I) to seven (Table 2.2).

2.2.3. Criteria used for selecting herbal prescriptions

In addition to the seven herbs chosen for the study, the use of multiple species prescriptions was also discussed with TCM practitioners. TCM relies on the synergy between herbs used in combination, sometimes with additional animal parts and/or minerals. Therefore, a literature survey was conducted for the TCM prescriptions containing bear bile. The aim of this exercise was to select prescriptions for investigation and to determine whether they demonstrate biological activity without bear bile. A major criterion for choosing TCM prescriptions was that they contained not more than one animal product (i.e. bear bile). It was also important to choose prescriptions that did not contain endangered plant species (those restricted by CITES). A TCM prescription found to comply with the criteria is Prescription X (Table 2.3), used in the treatment of laryngitis and containing bear bile with six herbs (Zhu, 1989).

Table 2.1. Properties and functions of bear bile and UDCA used as criteria for selection of single herbs for investigation.

Criteria	Properties and functions of bear bile and UDCA
a	'Cold' nature
b	'Bitter' taste
c	'Heat clearing'
d	'Fire purging'
e	Anti-inflammatory properties
f	Hepatoproctective properties
g	Anti-neoplastic properties
h	Cardiovascular protective properties

Criteria a – d: TCM properties of bear bile (Bensky and Gamble, 1993; Chang and But, 1987). Criteria e – h: Pharmacological / clinical effects of bear bile and UDCA reported in the scientific literature (Li *et al.*, 1995; Lee, 1999; Van Den Bogaert *et al.*, 2003; Im and Martinez, 2004).

Table 2.2. Seven herbs selected after consultation with TCM practitioners and from evaluation of TCM literature and pharmacological and clinical data.

Plant species with some similar properties to bear bile	a	b	c	d	e	f	g	h	Reference
1. Zhi zi, fruit of *Gardenia jasminoides* Ellis or *G. augusta* Merr. (Rubiaceae)	*	*	*	*	*	*	*		1, 2, 4
2. Zhi mu, rhizome of *Anemarrhena asphodeloides* Bge. (Anthericaceae)	*	*	*	*			*		1, 2, 4
3. Huang qin, root of *Scutellaria baicalensis* Georgi[+] (Labiatae)	*	*	*		*	*	*	*	1, 2, 4
4. Huang lian, rhizome of *Coptis chinensis* Franch.[+] (Ranunculaceae)	*	*	*		*	*	*	*	1, 2, 4
5. Huang bai, cortex of *Phellodendron amurense* Rupr. or *P. chinense* .Schneid. (Rutaceae)	*	*	*		*	*		*	1
6. Chuan xin lian, aerial part *of Andrographis paniculata* Nees (Acanthaceae)	*	*	*		*	*		*	1, 2, 4
7. Da huang, root and rhizome of *Rheum palmatum* L.[+] (Polygonaceae)	*	*	*		*	*			1, 3

+ Other plant species may also be used for the Chinese name specified (not listed in Table 2.2). Criteria a – d (refer to Table 2.1): based on TCM literature (Hsu et al., 1986[1]; Chang and But, 1987[2]; Chang and But, 2001[3]; Bensky and Gamble, 1993[4]). Criteria e – h (refer to Table 2.1): based on pharmacological and clinical data.

Table 2.3. Herbal composition of prescription X.

TCM name, plant part, pant species and family
Zhi zi, fruit of *Gardenia jasminoides* Ellis or *G. augusta* Merr. (Rubiaceae)
Huang lian, rhizome of *Coptis chinensis* Franch.[+] (Ranunculaceae)
Ban lan gen, root of *Isatis indigotica* Fort., *I. tinctoria* L. (Cruciferae) or *Baphicacanthus cusia* Bremek. (Acanthaceae)
Jin yin hua, flower bud of *Lonicera japonica* Thunb., *L. confusa* DC., *L. dasystyla* Rehd., or *L. hypoglauca* Miq. (Caprifoliaceae)
Lian qiao, fruit of Forsythia suspensa Vahl, F. viridissima Lindl. F. koreana Nakai (Oleaceae)
Hu po, resin of *Pinus succinifer* (Pinaceae)

+ Indicates that other plant species may be used for the Chinese name specified (not listed in Table 2.3).

In addition, two Chinese-Japanese (Kampo) patent medicines, orengedokuto and diao-orengedokuto are also proposed as possible

replacements for bear bile. Diao-orengedokuto is orengedokuto with an additional herb, Rhei Radix et Rhizoma and is used in the treatment of artherosclerosis (Kim *et al.*, 2002c). These two prescriptions were selected on the basis that they are composed of herbs proposed from this investigation as replacements for bear bile (Table 2.4), and also possess certain similar biological and TCM functions to bear bile.

Table 2.4. Composition of Orengedokuto

TCM names of herbs*
Huang qin (Scutellariae Radix)
Huang lian (Coptis Rhizoma)
Huang bai (Phellodendri Cortex)
Zhi zi (Gardenia Fructus)

*Sekiya *et al.*, 2002. Diao-orengedokuto is orengedokuto with an additional herb, Rhei Radix et Rhizoma (Kim *et al.*, 2002c).

2.3. Selection of Herbs to Investigate as Alternatives to Rhino Horn

2.3.1. Traditional uses of rhino horn

In TCM, rhino horn (*xi jiao*; Rhinocerotis Cornu) is considered to possess "the strongest action of 'clearing heat', 'removing heat from the blood', as well as arresting convulsions" (Zou, 1989). However, rhino horn has been reported to have low efficacy in reducing temperature in febrile animals (But *et al.*, 1990; Laburn and Mitchel, 1997). This could, in part, be explained by the differences in concepts of the pathology of fever between Western medicine and TCM. The major difference is that in TCM, febrile diseases are not necessarily accompanied by an increase in body temperature (Hsu *et al.*, 1986; Xu, 1994; Zou, 1989). In contrast, febrile diseases are associated with an increase in body temperature in Western medicine (Moltz, 1993).

Rhino horn is used in TCM as a detoxifying, anti-convulsant and anti-inflammatory agent. A major use is in the treatment of advanced stages of fever in the *ying* and blood conformation, complicated by delirium or coma. It is often used in combination with other TCM preparations and the horn is

reputed to be a potent anti-convulsant in these preparations (Zou, 1989). Haemorrhagic conditions (e.g. erythema, haematemesis and epistaxis) sometimes manifest symptoms associated with conditions treated by rhino horn. Rhino horn has also been associated with the treatment of cardiovascular diseases (Chang and But, 1987).

2.3.2. Criteria used for selecting single herbs and prescriptions

After consulting TCM practitioners, a set of criteria was developed for herb selection (Table 2.5). A survey of the TCM literature was carried out applying these criteria to identify herbs to be investigated as possible replacements for rhino horn. In further discussion with TCM practitioners, nine plant species (Table 2.6) were selected from those identified in the literature, for investigation to evaluate their suitability as possible alternatives to rhino horn.

When combined in prescriptions with TCM herbs, rhino horn is reputed to play an important role. Therefore, another literature survey was conducted to identify TCM prescriptions containing rhino horn which could be studied in biological assays with and without rhino horn. Five TCM prescriptions (described in Table 2.7) were selected on the basis of containing rhino horn as the only animal component. Also selected was a TCM prescription, zhi zi jin hua, composed only of herbs which was used as a TCM 'control'. The prescriptions were made up of a total of 23 herbs and one mineral (Table 2.7).

Table 2.5. Properties and functions of rhino horn used as criteria for herb selection.

Criteria	Properties and functions of rhino horn
a	'Cold' nature
b	'Bitter' taste
c	'Salty' taste
d	'Blood cooling'
e	'Heat clearing'
f	Anti-convulsant
g	Anti-inflammatory properties
h	Antipyretic (fever-reducing) properties
i	Reduce haemorrhage

Criteria a – i: TCM properties and functions of rhino horn (Hsu *et al.*, 1986; Chang and But, 1987; Bensky and Gamble, 1993).

Table 2.6. Nine herbs selected after consultation with TCM practitioners and from evaluation of TCM literature, pharmacological and clinical data.

Herbs with similar properties to rhino horn	a	b	c	d	e	f	g	h	i	Ref.
1. Xuan shen, root of *Scrophularia ningpoensis* Hemsl[+] (Scrophulariaceae)	*	*	*	*	*		*	*		1, 2
2. Sheng di huang, root of *Rehmannia glutinosa* Steud. (Scrophulariaceae)	*	*		*	*		*			1, 2
3. Mu dan pi, root of *Paeonia suffruticosa* Andr. (Paeoniaceae)	*	*		*	*	*	*			1, 2
4. Chi shao yao, root of *Paeonia lactiflora* Pall., *P. obovata* Maxim. or *P. veitchii* Lynch (Paeoniaceae)	*	*		*	*	*	*		*	1
5. Zi cao, root of *Arnebia euchroma* I.M.Johnst. or *Macrotomia euchromon* Paulsen (Boraginaceae)	*		*	*	*		*			1
6. Ban lan gen, root of *Isatis indigotica*, *I. tinctoria* L. (Cruciferae) or *Baphicacanthus cusia* (Acanthaceae)	*	*		*	*		*	*		1, 2
7. Jin yin hua, flower bud of *Lonicera japonica* Thunb. (Caprifoliaceae)		*		*	*	*	*			1, 2
8. Lian qiao, fruit of *Forsythia suspensa* Vahl, *F. viridissima* Lindl., *F. koreana* Nakai (Oleaceae)	*	*			*		*	*		1, 2
9. Dan shen, root of *Salvia miltiorrhiza* Bge (Lamiaceae)	*	*			*		*			1

Criteria a – i (refer to Table 2.5): based on TCM literature (Hsu *et al.*, 1986[1]; Bensky and Gamble, 1993[2]).

Table 2.7. The distribution of 23 herbs and one mineral in six TCM prescriptions.

	TCM control	TCM prescriptions traditionally containing rhino horn				
	Zhi zi jin hua	Qingwen baidu yin	Xi jiao dihuang tang	Qing ying tang	Sheng xi dan	Qing gong tang
1. Xuan shen, root of *Scrophularia ningpoensis*+ (Scrophulariaceae)		*		*	*	*
2. Sheng di huang, root of *Rehmannia glutinosa* (Scrophulariaceae)		*	*	*	*	
3. Mu dan pi, root of *Paeonia suffruticosa* Andr. (Paeoniaceae)		*	*			
4. Chi shao yao, root of *Paeonia lactiflora*, *P. obovata* or *P. veitchii* (Paeoniaceae)		*	*			
5. Zi cao, root of *Arnebia euchroma*+ (Boraginaceae)					*	
6. Ban lan gen, root of *Isatis indigotica*, *I. tinctoria* (Cruciferae) or *Baphicacanthus cusia* (Acanthaceae)					*	
7. Jin yin hua, flower bud of *Lonicera japonica* (Caprifoliaceae)	*			*	*	
8. Lian qiao, fruit of *Forsythia suspensa*+ (Oleaceae)		*		*	*	*
9. Dan shen, root of *Salvia miltiorrhiza* (Lamiaceae)				*		
10. Zhi mu, rhizome of *Anemarrhena asphodeloides* (Anthericaceae)	*	*				
11. Zhi zi, fruit of *Gardenia jasminoides* or *G. augusta* (Rubiaceae)	*	*				
12. Huang qin, root of *Scutellaria baicalensis*+ (Labiatae)	*	*			*	
13. Huang lian, rhizome of *Coptis chinensis*+ (Ranunculaceae)	*	*		*		
14. Huang bai, cortex of *Phellodendron amurense*+ (Rutaceae)	*					
15. Da huang, root and rhizome of *Rheum palmatum* L.+ (Polygonaceae)	*					
17. Tian hua fen, root of *Trichosanthes kirilowii* (Cucurbitaceae)	*				*	
18. Lian zi xin, seed of *Nelumbo nucifera* (Nelumbonaceae)						*
19. Mai men dong, root of *Ophiopogonis japonicus*+ (Liliaceae)				*		*
20. Dan zhu ye, aerial part of *Lophatherum gracile* (Poaceae)		*		*		*
21. Jie geng, root of *Platycodon grandiflorum* (Campanulaceae)		*				
21. Gan cao, root of *Glycyrrhiza uralensis* or *G. glabra*+ (Leguminosae)		*				
22. Chang pu, rhizome of *Acorus gramineus* (Acoraceae)					*	
23. Dan dou chi, seed of *Glycine max* (Leguminosae)					*	
24. Shi gao, calcium sulphate		*				

Zhi zi jin hua was used as a TCM control prescription in biological tests. The herbs with bold lettering are also listed in Table 2.6 as possible herbal alternatives to rhino horn.

2.3.2.1. Reputed and demonstrated effects of prescriptions containing rhino horn

Qing ying tang is a prescription for 'clearing heat' in the 'ying' system and contains eight herbs (Table 2.7) in addition to rhino horn (Zhu, 1989; Xu, 1994; Zou, 1989). The combined horn-herb extracts and the prescription without animal product were both found to be antipyretic in rats (But and Tam, 1991).

The prescription qingwen baidu yin is reputed to possess antipyretic and anti-toxic properties and is used to 'clear away' heat from 'qi' and blood systems (Xu, 1994). The prescription contains 12 herbs, one mineral (Table 2.7) and rhino horn. Five of the herbs are also included in the qing ying tang prescription (Table 2.7). A modified version of qingwen baidu yin, with buffalo horn substituted for rhino horn, was found to be effective in reducing fever in rabbits, but the prescription without added animal product was not tested (Xie, 1993).

Xi jiao dihuang tang is a prescription used to 'clear away' heat from 'qi' and blood systems (Zhu, 1989; Bensky and Barolet, 1990; Xu, 1994; Zou, 1989). It contains rhino horn (the principal component) and three herbs, as described in Table 2.7. All four components of xi jiao dihuang tang are also included in qingwen baidu yin (Table 2.7).

Sheng xi dan is also known as 'magical rhinoceros special pill' used to treat febrile diseases (Bensky and Barolet, 1990). It is composed of rhino horn with 10 herbs (Table 2.7). Qing gong tang is used in the treatment of epidemic febrile diseases (Zou, 1989) and consists of rhino horn with five herbs (Table 2.7). Zhi zi jin hua is listed in the Pharmacopoeia of the People's Republic of China (1997) as an antipyretic agent and is composed of eight herbs (Table 2.7).

2.4. Concluding Remarks

Ten different herbs both as single species and included as constituents of herbal prescriptions, (Tables 2.2 and 2.3) were selected for the bear bile project. In addition a resin of *Pinus succinifer* (Pinaceae) formed part of Prescription X (Table 2.3) studied as part of the bear bile project. In total 23 herbs (Table 2.7; numbers 1-23) and one preparation derived from a calcium sulphate (Table 2.7, number 24) formed part of the prescriptions investigated as part of the rhino horn project. It is apparent from Table 2.7, that all nine possible herbal 'replacements' for rhino horn (Table 2.6) also exist in one or more of these prescriptions. This finding confirms the practice in TCM of combining herbs with similar functions for their additive and synergistic effects. Nine of the selected herbs (Table 2.7; herbs 6-8, and 10-15) overlapped with the bear bile and the rhino horn project.

The 24 herbs selected were prepared (Chapter 4) for activity tests (Chapters 5, 6 and 7). Prior to these activity tests, analyses were conducted to determine the quality of the herb samples obtained (Chapter 3).

CHAPTER 3. CHEMICAL METHODS TO EVALUATE THE QUALITY OF HERBS

3.1. Introduction

The quality and safety of herbal medicines is of concern and this has prompted strong support for the statutory regulation of herbal medicines and practitioners in the UK (Directive 2004/24/EC; Department of Health, 2005). As well as concern over the safety of herbal preparations (Rousseaux and Schachter, 2003), conservation organisations have expressed concern relating to the overexploitation of plant and animal species (CITES, 2006). Occasional cases of toxicity have occurred due to the mis-identification of plant species or deliberate substitution with herbs which may be toxic (Gafner *et al.*, 2003). Therefore, it was important that prior to testing in biological assays, the plant species of the herbs were identified and verified.

In addition to the issues relating to the identity of plant species, some herbs have been reported to be contaminated with micro-organisms, heavy metals, pesticide residues or pharmaceutical drugs (Koh and Woo, 2000; Dahl, 2001). In biomedical research, contamination of herbs (with e.g. metals and pesticide residues) could interfere with certain biological activity tests, leading to false positives or negatives. In the present study, the biological activity tests which were conducted include nuclear factor kappaB (Chapter 6) and cytochrome P450s (Chapter 7) activities. Cytochrome P450 enzymes are responsible for the metabolism of several endogenous and exogenous compounds including pesticides, pharmaceutical drugs and herbs. Metals such as Cd and Hg are also modulators of several cytochrome P450 isoforms (Alexidis *et al.*, 1994; Maier *et al.*, 2000). Metal modulators of the nuclear factor kappaB include Zn (Zhou *et al.*, 2004), V (Huang *et al.*, 1998), Mn (Ramesh *et al.*, 2002), Ni (Cruz *et al.*, 2004), Pb (Zuscik *et al.*, 2002), Hg (Kim *et al.*, 2002b), Fe (Xiong *et al.*, 2003), Cr (Ding and Shi, 2002), Co (Sultana *et al.*, 1999), Cd (Jeong *et al.*, 2004), Be (Ding *et al.*, 2000) and Al (Campbell *et al.*, 2002).

In the present study, all of the 14 metals listed above were included in a list of 16 metals determined in selected herbs. Multi-residue pesticide analyses were also conducted. In these preliminary analyses, the main aim was to

determine the concentrations of metals and pesticide residues in the herbs. However, in order to make a direct correlation between the biological activities of the herbs and levels of contaminants, further studies would be required.

3.2. Identification of Plant Species

Chemical, anatomical (Then *et al.*, 1998) or genetic (Crockett *et al.*, 2004) characteristics can be used to confirm the identification of a plant species. This process is often referred to as authentication. A commonly used analytical method to obtain chemical profiles of plant species is thin layer chromatography (Pharmacopoeia of the PRC, 1997). High performance liquid chromatography coupled to a diode array detector (HPLC-DAD) is also often used, for example, for the identification of *Ephedra sinica* (Schaneberg *et al.*, 2003) and *Ocimum americanum* (Viera *et al.*, 2003). Liquid chromatography-mass spectrometry (LC-MS) has been used to successfully differentiate between ginseng species (Kite *et al.*, 2003) and to authenticate saponin extracts of *Quillaja saponaria* Molina (Kite *et al.*, 2004). Chemical fingerprinting using nuclear magnetic resonance (NMR) (Qin *et al.*, 2001) and Fourier transform Raman spectroscopy (Liu *et al.*, 2002) has also been reported for this authentication process. In this study, the HPLC (UV-DAD) profiles of phenolic compounds in herb samples were used as chemical fingerprints to aid in the identification of the plant species. The work was conducted in collaboration with the Biological Interactions Section of the Jodrell Laboratory, RBG, Kew.

3.2.1. Phenolic compounds

Phenolic compounds (phenolics) are one of the largest groups of phytochemicals (Markham, 1982). Flavonoids include about 8000 natural compounds (Pietta, 2000) making them the largest class of compounds within the phenolic group. Some of the various modifications of the flavonoid structure which can occur are summarised in Fig. 3.1.

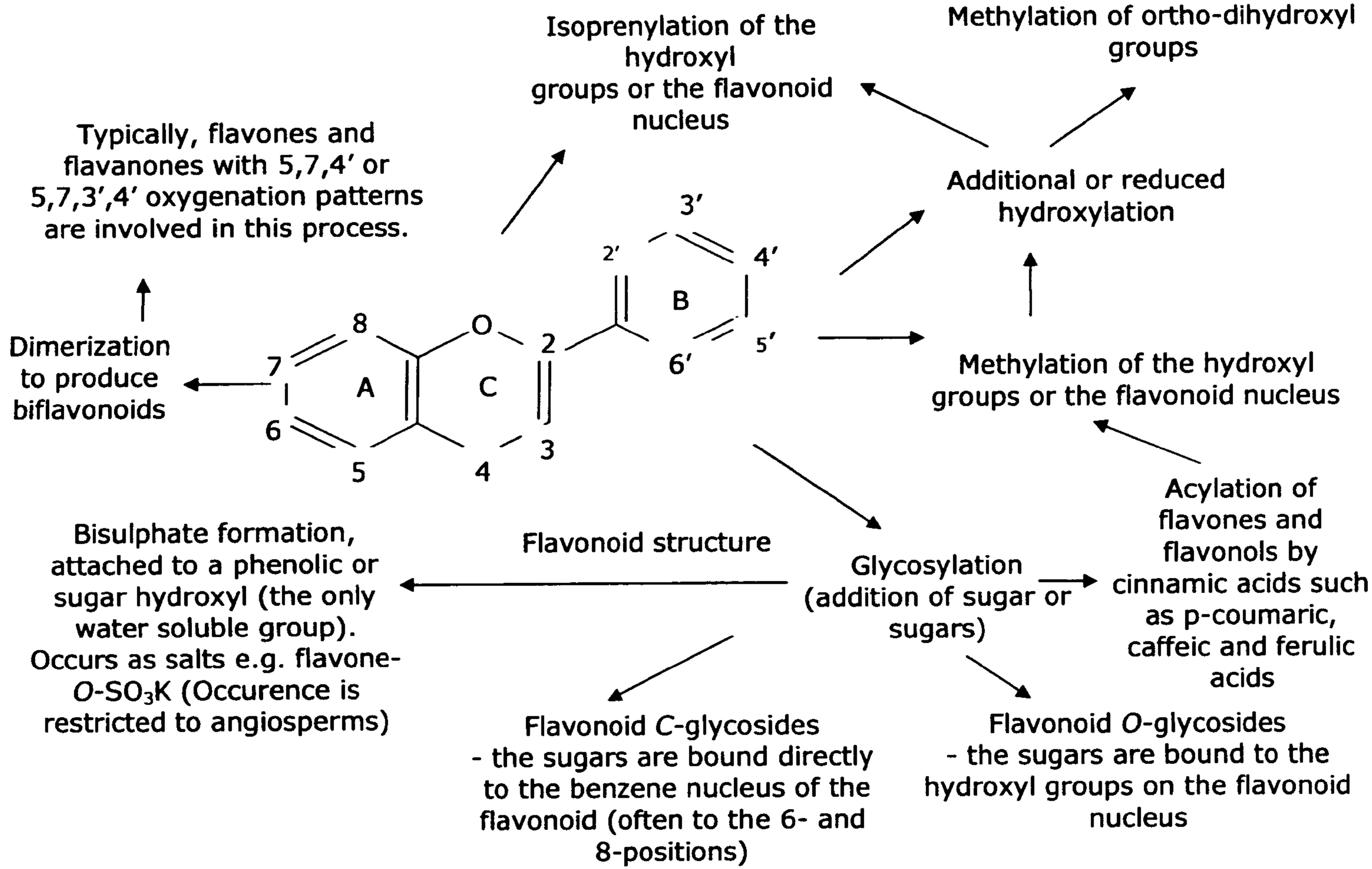

Fig. 3.1. Modifications of the flavonoid structure: hydroxylation, methylation, glycosylation and dimerisation (summarized from Markham, 1982).

Changes in the flavonoid structure can significantly affect their chemical profiles as indicated by ultraviolet (UV) spectroscopy and retention times in HPLC and some of these effects are given in Appendices III and IV, respectively. Several classes of phenolics show distinct UV profiles (Ferreres *et al.*, 1989; Grayer *et al.*, 2001) and some published reference UV spectra (Harborne *et al.*, 1975, Markham, 1982; Dey and Harborne, 1989) are summarized in Appendix V.

3.2.2. Experimental procedure

3.2.2.1. Samples

Samples of TCM material were kindly donated by TCM suppliers, Jo Liu of Mayway (UK) Ltd. (Hanwell) and Paul Skipworth of Kingham Herbs and Tinctures (UK). A total of 55 trade samples were analysed to enable comparison of their chemical profiles. Twenty four trade samples were later tested for biological activity. To identify the plant species, reference samples of TCM material were obtained from verified herbarium voucher specimens at Royal Botanic Gardens, Kew. Information about the plants used in this study is given in Appendix VI.

3.2.2.2. Extraction

TCM materials (trade samples and reference materials) were ground using a pestle and mortar or grinding equipment. Ground material (0.5 g) was then extracted overnight using 80% aqueous methanol (5 ml) at room temperature. Following extraction, extracts were filtered through Whatman filters (# 1) under reduced pressure, using a Buchner flask. The filtrate was evaporated to dryness and reconstituted in methanol prior to HPLC analysis. The remaining extracts were transferred to pre-weighed vials, left to dry in a fume cupboard and stored below 5^{o}C.

3.2.2.3. HPLC (UV-DAD) method

Analytical HPLC was carried out using equipment incorporating a Waters LC600 pump and a 996 photodiode array detector (Grayer and de Kok, 1998). A LiChrosphor 100RP-18 (250 x 4.0mm i.d.; 5µm particle size) column (maintained at 30°C) was used for analysis with an eluent flow rate of 1ml/min. The mobile phase consisted of 2% aqueous acetic acid (A) and methanol/acetic acid/water (18:1:1) (B). Initial conditions were 75% A and 25% B and the proportion of B was increased according to a linear gradient, reaching 100% B after 20 minutes. This was followed by an isocratic elution with 100% B for a further 5 minutes and return to start conditions after 10 minutes. Injections (10 – 40 µl) were made by an autosampler. The UV-visible spectra (210-400 nm) were recorded on-line during the HPLC analysis.

3.2.3. Results

The UV profiles and retention times from HPLC (UV-DAD) obtained for samples were cross-referenced to reference materials. The plant species were identified for six out of the seven herbs investigated as alternatives for bear bile (Table 3.1 and Appendix VIIa) and seven out of nine herbs investigated as alternatives for rhino horn (Table 3.2 and Appendix VIIb). The species of four out of the eight remaining herbs found in the TCM prescriptions were verified (Table 3.3). As an example, the chromatograms obtained for the analysis of three trade samples of Gardenia Fructus, compared to authentic samples of *Gardenia jasminoides* and *G. augusta* are given in Figs. 3.2 and 3.3, respectively.

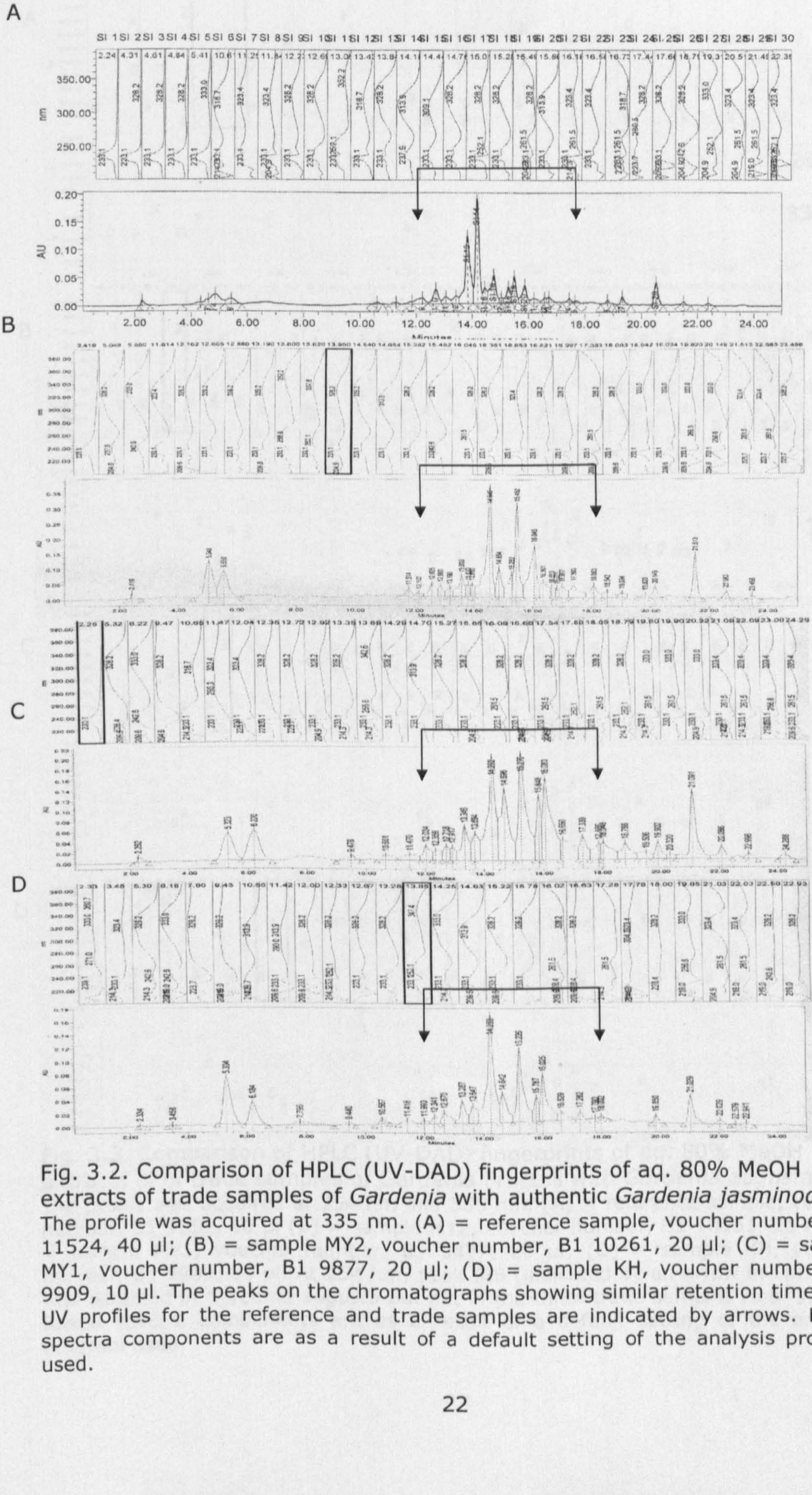

Fig. 3.2. Comparison of HPLC (UV-DAD) fingerprints of aq. 80% MeOH extracts of trade samples of *Gardenia* with authentic *Gardenia jasminodes*. The profile was acquired at 335 nm. (A) = reference sample, voucher number, B1 11524, 40 µl; (B) = sample MY2, voucher number, B1 10261, 20 µl; (C) = sample MY1, voucher number, B1 9877, 20 µl; (D) = sample KH, voucher number, B1 9909, 10 µl. The peaks on the chromatographs showing similar retention times and UV profiles for the reference and trade samples are indicated by arrows. Boxed spectra components are as a result of a default setting of the analysis program used.

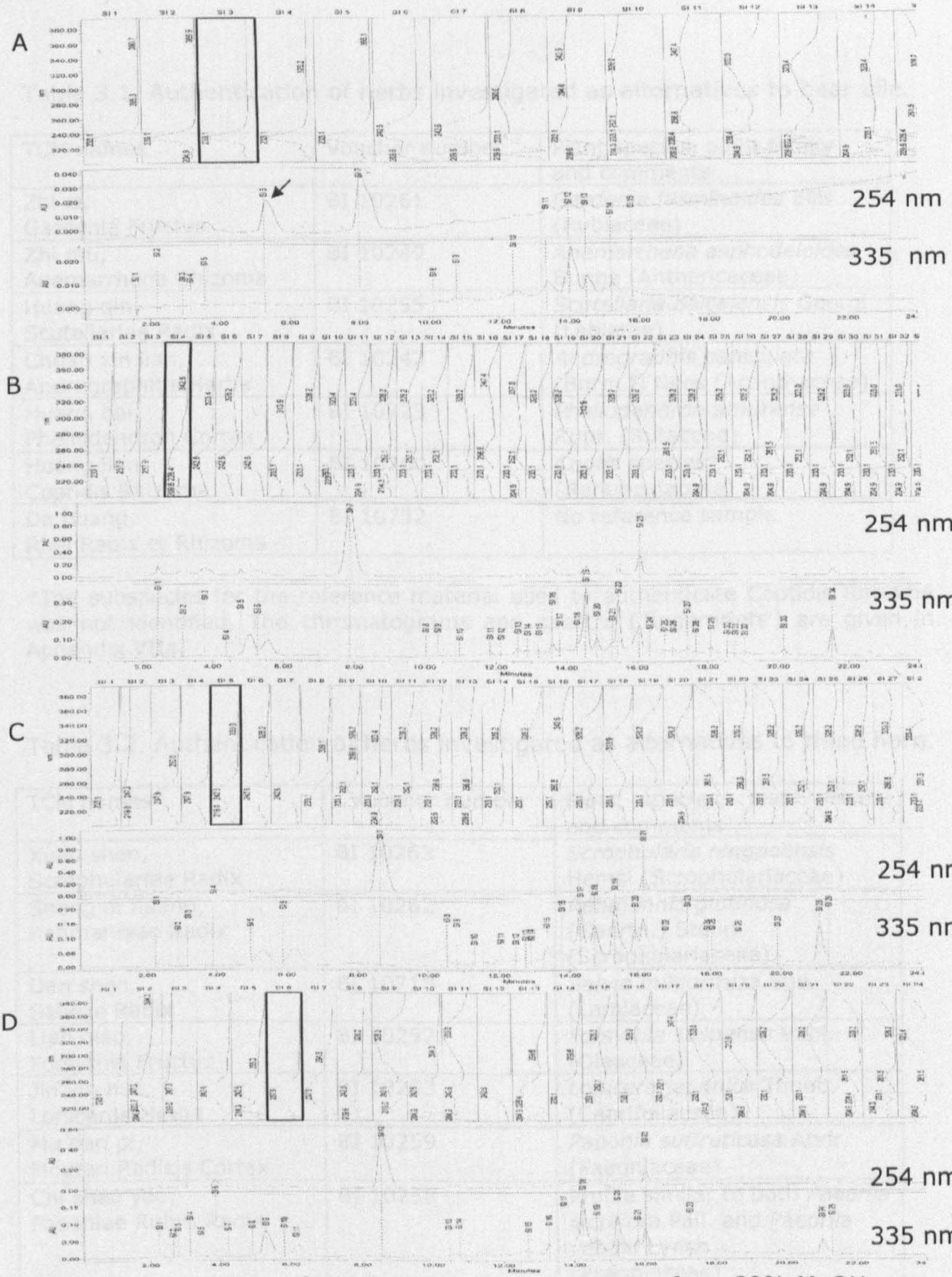

Fig. 3.3. Comparison of HPLC (UV-DAD) fingerprints of aq. 80% MeOH extracts of of trade samples of Gardenia Fructus with *Gardenia augusta*. The profile was acquired at 254 nm and 335 nm. (A) = reference sample, voucher number, B1 9951, 10 µl (B) = sample MY2, voucher number, B1 10261, 20 µl; (C) = sample MY1, voucher number, B1 9877, 20 µl; (D) = sample KH, voucher number, B1 9909, 10 µl. At 254 nm, peak SI6 (indicated by an arrow in Fig. 3.3A) was detected in *Gardenia augusta* but not in the three trade samples Figs. 3.3B-D. Boxed spectra components are as a result of a default setting of the analysis program used.

Table 3.1. Authentication of herbs investigated as alternatives to bear bile.

TCM names	Voucher number	Plant species, plant family and comments
Zhi zi, Gardenia Fructus	BI 10261	*Gardenia jasminoides* Ellis (Rubiaceae)
Zhi mu, Anemarrhena Rhizoma	BI 10249	*Anemarrhena asphodeloides* Bunge (Anthericaceae)
Huang qin, Scutellariae Radix	BI 10255	*Scutellaria baicalensis* Georgi (Labiatae)
Chuan xin lian, Andrographitis Herba	BI 10242	*Andrographis paniculata* (Burm.f) Nees (Acanthaceae)
Huang bai, Phellodendron Cortex	BI 10723	*Phellodendron amurense* Rupr. (Rutaceae)
Huang lian, Coptidis Rhizoma	BI 10260	*Coptis* species* (Ranunculaceae)
Da huang, Rhei Radix et Rhizoma	BI 10732	No reference sample

*The subspecies for the reference material used to authenticate Coptidis Rhizoma was not identified. The chromatograms and spectra ('fingerprints') are given in Appendix VIIa.

Table 3.2. Authentication of herbs investigated as alternatives to rhino horn.

TCM names	Voucher number	Plant species, plant family and comments
Xuan shen, Scrophulariae Radix	BI 10263	*Scrophularia ningpoensis* Hemsl (Scrophulariaceae)
Sheng di huang, Rehmanniae Radix	BI 10262	*Rehmannia glutinosa* (Gaertn.) Steud. (Scrophulariaceae)
Dan shen, Salviae Radix	BI 10718	*Salvia miltiorrhiza* Bge. (Lamiaceae)
Lian qiao, Forsythia Fructus	BI 10257	*Forsythia suspensa* Vahl. (Oleaceae)
Jin yin hua, Lonicerae Herba	BI 10253	*Lonicera japonica* Thunb. (Caprifoliaceae)
Mu dan pi, Moutan Radicis Cortex	BI 10259	*Paeonia suffruticosa* Andr. (Paeoniaceae)
Chi shao yao, Paeoniae Rubra Radix	BI 10258	Profile similar to both *Paeonia lactiflora* Pall. and *Paeonia vetchii* Lynch (Paeoniaceae)
Ban lan gen, Isatidis Radix	BI 10251	Species not confirmed
Zi cao, Arnebiae seu Lithospermi Radix	BI 10724	Species not confirmed

The chromatograms and spectra ('fingerprints') are given in Appendix VIIb.

Table 3.3. Authentication of other herbs in TCM prescriptions.

TCM names	Voucher number	Plant species, plant family and comments
Dan zhu ye Lophatheri Herba	BI 10254	*Lophatherum gracile* Brongn. (Poaceae)
Jie geng Platycodi Radix	BI 10252	*Platycodon grandiflorum* (Jacq.) A.DC. (Campanulaceae)
Gan cao Glycyrrhizae Radix	B1 10256	Profiles indicate sample is a species of *Glycyrrhiza* and profile is similar to *G. uralensis* Fisch. (Leguminosae)
Mai men dong Ophiopogonis Radix	BI 10758	*Ophiopogon* species Liliaceae
Chang pu Acori Graminei Rhizoma	BI 10741	No reference sample
Dan dou chi Sojae Praeparata semen	BI 10734	No reference sample
Lian zi xin Nelumbinis Nucifera Plumula	BI 10757	No reference sample
Tian hua fen Trichosanthis Radix	BI 10721	No reference sample

3.2.4. Discussion

A TCM name for a herb may refer to more than one plant species, for example Scutellaria Radix (huang qin) is prepared from the root of *Scutellaria baicalensis* Georgi, *S. viscidula* Bge., *S. amoena* Wright, *S. ikonnikovii* Juz., *S. rehderiana* Diel, *S. hypericifolia* H.Lév. or *S. likiangensis* Diels (Chang and But, 1987) from the Labiatae plant family. Verification of the TCM material is essential to ensure that when interpreting data regarding the chemistry and pharmacological activities of each species, the results refer to the correct plant species. Thus, when recommending potential alternatives to the animal material (bear bile and rhino horn), it was important that the correct plant species were proposed as alternatives.

Results for the herb Gardenia Fructus (zhi zi) are used as an example to illustrate the interpretation of HPLC (UV-DAD) chromatograms and spectra obtained in this study. Gardenia Fructus can be prepared from either *Gardenia jasminoides* Ellis or *G. augusta* Merr. When the chromatograms for

three trade samples were compared to the authentic sample, the profiles of the trade samples exhibited similarities to both species of *Gardenia* at 335 nm, particularly between peaks eluting at 12 – 18 minutes (Figs. 3.2 and 3.3). However, at 254 nm, a characteristic peak, (labelled SI6, retention time about 5.3 min) in *G. augusta* (Fig. 3.3A) was not pronounced in any of the three trade samples (Figs. 3.3B to D) or in *G. jasminoides*. In addition, a certificate of analysis provided by the supplier of one of the trade samples (MY1) stated *G. jasminoides* as the source of plant material. The samples MY1 and MY2 were obtained from the same herb supplier but at different times, however, they shared the same batch number (Appendix VI) and similar HPLC profile (Figs. 3.2 B & C and 3.3B & C). Therefore, for the purposes of this project, HPLC provided sufficient information to indicate that the plant species was as described in TCM, and likely to be *G. jasminoides*. For a more definite differentiation of plant species, other identification methods may be required. Of the 55 individual trade samples analysed, certificates of analysis were obtained from the suppliers for only four of the herbs.

The main objective of the current study was to identify the different plant species and initially not to obtain information about the presence of specific compounds in the extract that might be associated with their activity. However, reference UV absorption maxima for phenolic compounds (Appendix V) have been used to aid the identification of compounds (Appendix VII). The presence of some of these compounds serves as further confirmation of the plant species. Flavonoids such as flavones and flavonols can sometimes be modified by acylation with cinnamic acids (e.g. *p*-coumaric, caffeic and ferulic acids) (Fig. 3.1) and the products show distinctive UV profiles in MeOH dominated by a wide maxima in the UV range, 310-330 nm (Ferreres *et al.*, 1989). Caffeic acid derivatives were detected in several plant species, including *Andrographitis paniculata*, *Phellodendron amurense*, *Salvia miltiorrhiza*, *Forsythia suspensa*, *Scrophularia ningpoensis* (verbascoside-type) and *Rehmannia glutinosa* (verbascoside-type) (Appendix VII).

To aid the authentication of plant species, marker compounds (irrelevant to their biological activity) can be identified. However, in some cases the most prominent peaks may not be suitable in identifying plant species in the absence of an authentic sample. Although marker compounds are useful in the identification and authentication of several plant species, the presence of a marker compound in a product may not guarantee the presence of a particular species. Some prepared herbs may be 'spiked' with a marker compound (Schaneberg *et al.*, 2003) or may contain a substituted plant species which contains the marker compound. Also, batch variations may present variations in the composition, as well as the profile of some chemicals in plants (Dong *et al.*, 2003).

Since HPLC-DAD uses a UV detection system, it is not suitable for the identification of many of those compounds which do not possess strong UV absorption characteristics. For example, this is the situation for many diterpenoids, fatty acids, alkaloids and some volatile compounds. Other analytical methods which may be used to differentiate between plant species include LC-MS (Kite *et al.*, 2004), NMR (Qin *et al.*, 2001) and Fourier Transform Raman spectroscopy (Liu *et al.*, 2002).

Batch variations in the phytochemical profiles of plant material are often encountered due to genetic variations as well as factors such as differences in climate, soil, collection times and methods of preparation. This presents a particularly challenging task in the assessment of the quality of the herbs. Another challenge in the authentication of Chinese herbs is the availability of authentic samples in the UK. As an illustration, Isatidis Radix can be prepared from *Baphicacanthus cusia* Bremek (Acanthaceae), *Isatis indigotica* Fort. or *I. tinctoria* L. (Cruciferae). An authentic sample was available only for *I. indigotica* and the HPLC profile was significantly different from those of the trade samples. The profiles of the three trade samples were, however, comparable to each other. In this instance it could only be concluded that the herbs were not prepared from *I. indigotica*. Another important factor in authentication is the reliability of the method used to authenticate the reference materials. Many of the reference materials used within this study were from the TCM collection at Kew which

originated in China as part of an international collaboration with the Institute of Medicinal Plant Development (IMPLAD), Beijing. However, the collection is not complete and further voucher samples are needed to enable all the possible plant substitutes to be studied.

To differentiate between the authenticated and non-authenticated herbs, this thesis now uses Latin names (in italics) for 17 herbs for which the plant species have been verified whereas TCM names are used for the other 7 herbs. In addition to authentication, aspects of herb safety and quality were assessed by determining concentrations of metal and pesticide residues (Sections 3.3 and 3.4, respectively).

3.3. Quantitative Analysis of Metals

There are different instrumental methods of analysis available for the determination of metals in foods and herbal preparations. Commonly used analytical techniques are atomic absorption spectrometry (Chuan *et al.*, 2000; Lozak *et al.*, 2002) and inductively coupled plasma (ICP) coupled with mass spectrometry (Ward and Savage, 1994, Lozak *et al.*, 2002), optical emission spectrometry (OES) or atomic emission spectrometry (Ward and Savage, 1994). An alternative method using neutron activation analysis is a convenient method for analysing trace elements in different matrixes (Sarmani *et al.*, 1999). The ICP-OES technique is often used to determine concentrations of various elements (including Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Si, and Zn) with good precision (Ward and Savage, 1994; Coscione *et al.*, 2002; Costa *et al.*, 2002). In this study, ICP-OES was used to determine the concentrations of metals in TCM samples.

3.3.1. Experimental procedure

3.3.1.1. Samples

The range of TCM herbs described Table 3.4 and rhino horn were analysed for metals. The table also identifies the part of plant from which the samples were collected.

Table 3.4. Samples analysed for metals and pesticide residues.

Plant species and Pin yin name	Plant part	Code
Forsythia suspensa Vahl (lian qiao)	Fruit	LQ
Gardenia jasminoides Ellis (zhi zi)	Fruit	ZZ
Lonicera japonica Thunb. (jin yin hua)	Flower bud	JYH
Lophatherum gracile Brongn. (dan zhu ye)	Aerial part	DZY
**Andrographis paniculata* Nees (chuan xin lian)	Aerial part	CXL
*Isatidis Folium (da qing ye)	Aerial part	DQY
Glycyrrhiza uralensis Fisch. (gan cao)	Root	GC
Paeonia suffruticosa Andr. (mu dan pi)	Root	MDP
Platycodon grandiflorum A.DC. (jie geng)	Root	JG
Rehmannia glutinosa Steud. (sheng di huang)	Root	SDH
Scutellaria baicalensis Georgi (huang qin)	Root	HQ
Scrophularia ningpoensis Hemsl. (xuan shen)	Root	XS
Paeonia lactiflora Pall. (chi shao yao)	Root	CSY
Isatidis Radix (ban lan gen)	Root	BLG
Coptis Rhizoma (huang lian)	Rhizome	HL
Anemarrhena asphodeloides Bge. (zhi mu)	Rhizome	ZM

* Samples not tested for pesticide residues.

3.3.1.2. Mixed calibration standards

Stock solutions (1000 µg/ml; matrix, 0.5 M nitric acid) of Al, Be, Cd, Co, Cr, Cu, Fe, Pb, Mn, Hg, Ni, Se, Ag, Sn, V, and Zn were prepared. This was used to prepare two different mixed metal standard solutions (1 µg/ml; in nitric

acid) as identified in Table 3.5 which also lists the reference detection wavelengths and the calculated limits of detection.

Table 3.5. Detection wavelengths and limits of detection (LOD) for metals in mixed stock solutions.

Mixed stock solution A			Mixed stock solution B		
Metal	Wavelength (nm)	LOD (µg/g)	Metal	wavelength (nm)	LOD (µg/g)
Ag	328.07	0.250	Al	396.15	0.058
Cu	325.8	0.070	Mn	257.61	0.028
Be	313.04	0.065	Fe	239.56	0.123
V	309.3	4.834	Pb	220.35	0.034
Co	238.1	0.073	Zn	213.86	0.114
Ni	221.6	0.077	Cd	215.44	0.038
Hg	195.2	0.168	Cr	205.6	0.027
Sn	189.99	0.036	Se	196.09	0.063

The LOD for each analysed metal was calculated as three times the standard deviation obtained from analysis of the procedural blank sample at a specified wavelength.

3.3.1.3. Extraction

Duplicate samples of dried herbs (10 g) were separately digested in glass beakers using excess nitric acid (16M; 30 ml) on a sand-bath (18^{o}C) to dryness. The extracts were re-dissolved in aqueous nitric acid (1.6M), filtered through Whatman filter paper (# 1) and each extract made up to a final volume of 50 ml. In a different set of experiments, rhino horn (1 g) was also extracted in nitric acid as described above. Each extract was analysed using ICP-OES procedure described in Section 3.3.1.4. Samples of *Glycyrrhiza uralensis* (gan cao) were extracted with nitric acid using either Teflon or glass beakers to assess beaker construction material.

3.3.1.4. ICP-OES method

A Perkin Elmer Plasma Model 40 ICP-OES was used to obtain triplicate readings of each procedural blank sample (1.6M nitric acid solution), the metal standards and each herb extract. In a separate experiment, six

replicate readings were obtained for the rhino horn extract. The limit of detection for each analysed metal was calculated as three times the standard deviation obtained from analysis of the blank sample at the specified wavelength for each metal (Table 3.5).

3.3.2. Results

3.3.2.1. Quality assessment experiments to assess beaker construction material

In order to evaluate if glass beakers were suitable for the extraction procedure, some samples were extracted using either Teflon or glass beakers and the results were compared. The results showed that Hg, Be, Co and Ag were all detected below their LODs for extractions using both Teflon and glass beakers (Table 3.6). However, extractions using glass beakers resulted in lower values of Sn, Cu and Ni being detected with a difference of 0.51 µg/g, 0.78 µg/g, 1.08 µg/g, respectively compared to Teflon beakers. Vanadium was the exception with a measured concentration 0.35 µg/g higher for the Teflon beakers in comparison with the glass beakers. For the purpose of this research, this preliminary analysis provided sufficient evidence that glass beakers could be used in the extraction procedure.

Table 3.6. Results of the quality assessment experiments to assess beaker construction material.

Metal	Concentration ± SD, µg/g (Teflon beaker)	Concentration ± SD, µg/g (Glass beaker)
Hg	none detected	none detected
Be	none detected	none detected
Co	none detected	none detected
Ag	none detected	none detected
Cu	2.69 ± 0.02	1.91 ± 0.06
Ni	3.05 ± 0.05	1.97 ± 0.15
V	3.86 ± 0.56	4.21 ± 0.49
Sn	4.54 ± 0.01	4.03 ± 0.21

3.3.2.2. Results for the metal analysis of rhino horn and TCM herbs

The results obtained for the metal analysis of rhino horn are summarised in Table 3.7. The results for the metal analysis of 16 herbs are shown diagrammatically in Figures 3.4 to 3.10. Four metals, Ag, Be, Co, and Hg were not detected in any of the herbs above their LODs of 0.25, 0.07, 0.07 and 0.17 µg/g, respectively. Vanadium (LOD, 4.8 µg/g) was detected in only *Andrographis paniculata* (9.9 ± 0.18 µg/g), Isatidis Radix (5.2 ± 0.01 µg/g), Isatidis Folium (14.5 ± 10.08 µg/g) and *Scutellaria baicalensis* (18.32 ± 0.43 µg/g).

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Table 3.7. Results for metal analysis of rhino horn.

Metal	LOD (µg/g)	Concentration in rhino horn ± SD (µg/g)	Legal limit in food (µg/g)
Ag	0.150	none detected	
V	0.288	none detected	
Sn	0.064	8.3 ± 1.2	200 (Food Regulations, 1992)
Pb	1.052	25.0 ± 3.0	10 (Chuan *et al.*, 2000)
Cd	0.047	0.6 ± 0.13	0.3 (Chuan *et al.*, 2000)
Zn	0.033	69.8 ± 0.08	50 (MAFF, 1956)
Cu	0.039	4.0 ± 0.11	20 (MAFF, 1956)
Hg	0.030	2.3 ± 1.4	0.5 (Lide, 1997; Chuan *et al.*, 2000)
Co	0.040	2.7 ± 0.78	
Be	0.009	0.3 ± 0.00	
Cr	0.024	0.5 ± 0.37	
Ni	0.166	4.7 ± 0.52	
Se	0.047	8.6 ± 2.9	
Mn	0.058	7.6 ± 0.08	
Al	0.164	235.6 ± 5.0	
Fe	0.093	321.4 ± 9.7	

Data represents the mean from six replicate readings ± SD.

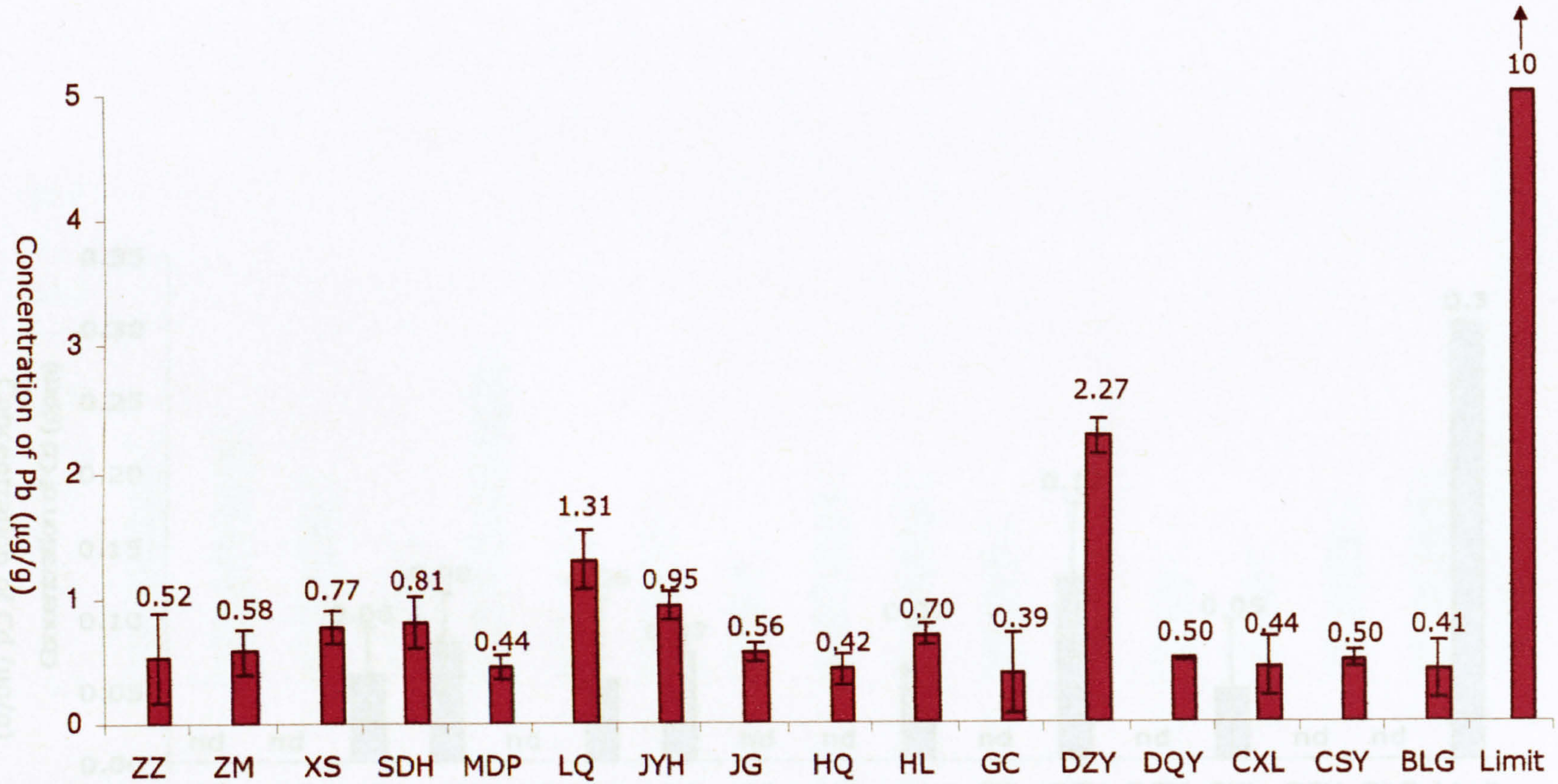


Fig. 3.4. Average concentrations of Pb in 16 herbs together with standard deviations (± SD).
The LOD for Pb in this study was 0.03 μg/g. In China the maximum permitted concentration for Pb (in dried herbs) is 10 μg/g (Chuan *et al.*, 2000).

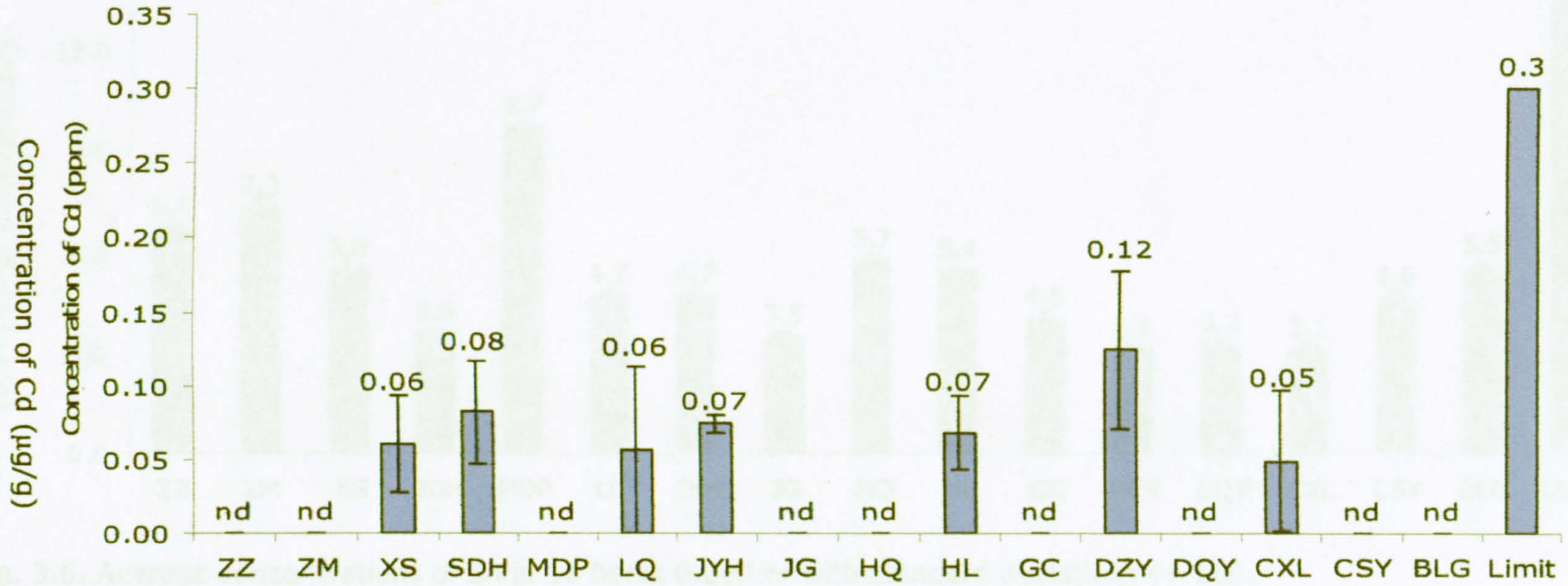


Fig. 3.5. Average concentrations of Cd in 16 herbs together with standard deviations (± SD).
The LOD for Cd in this study was 0.04 μg/g; nd = not detected above LOD. In China, the current maximum permitted concentration for Cd in food is 0.3 μg/g (Chuan *et al.*, 2000).

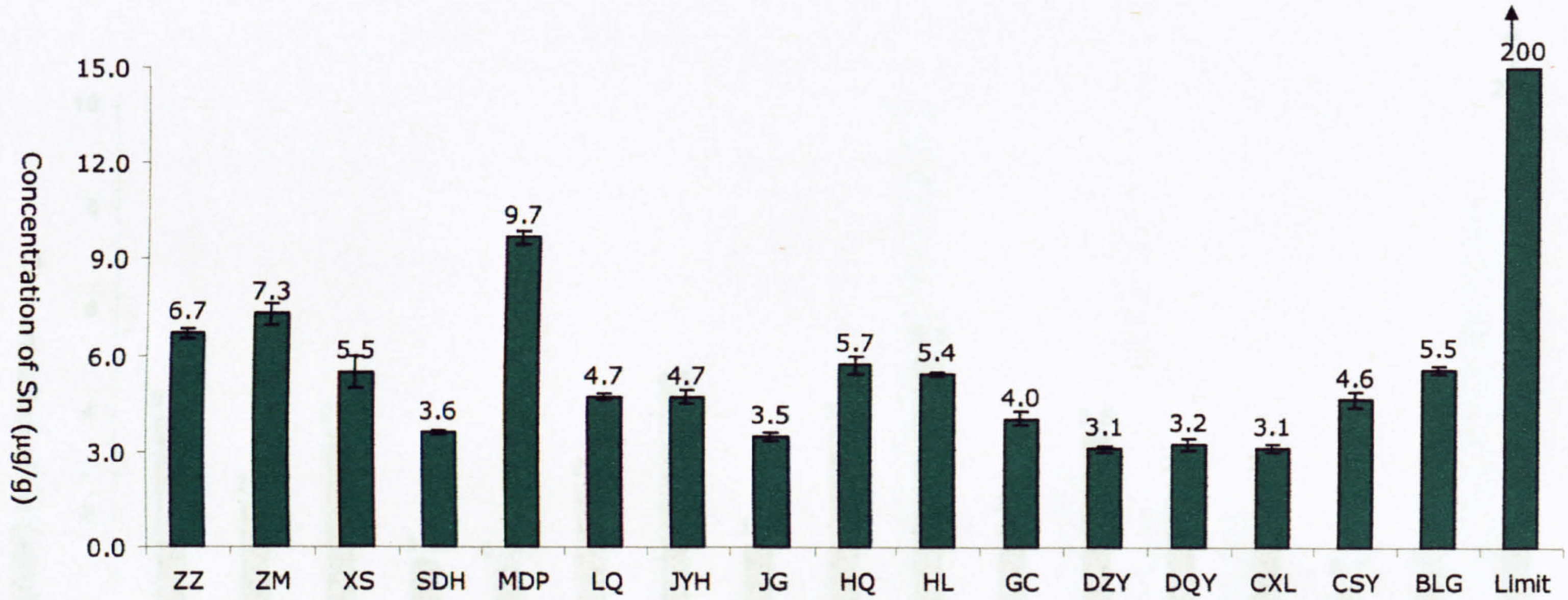


Fig. 3.6. Average concentrations of Sn in 16 herbs together with standard deviations (+ SD).
The LOD for Sn in this study was 0.04 μg/g. The UK statutory limit of 200 μg/g for Sn in food was set by the Food Regulations (1992).

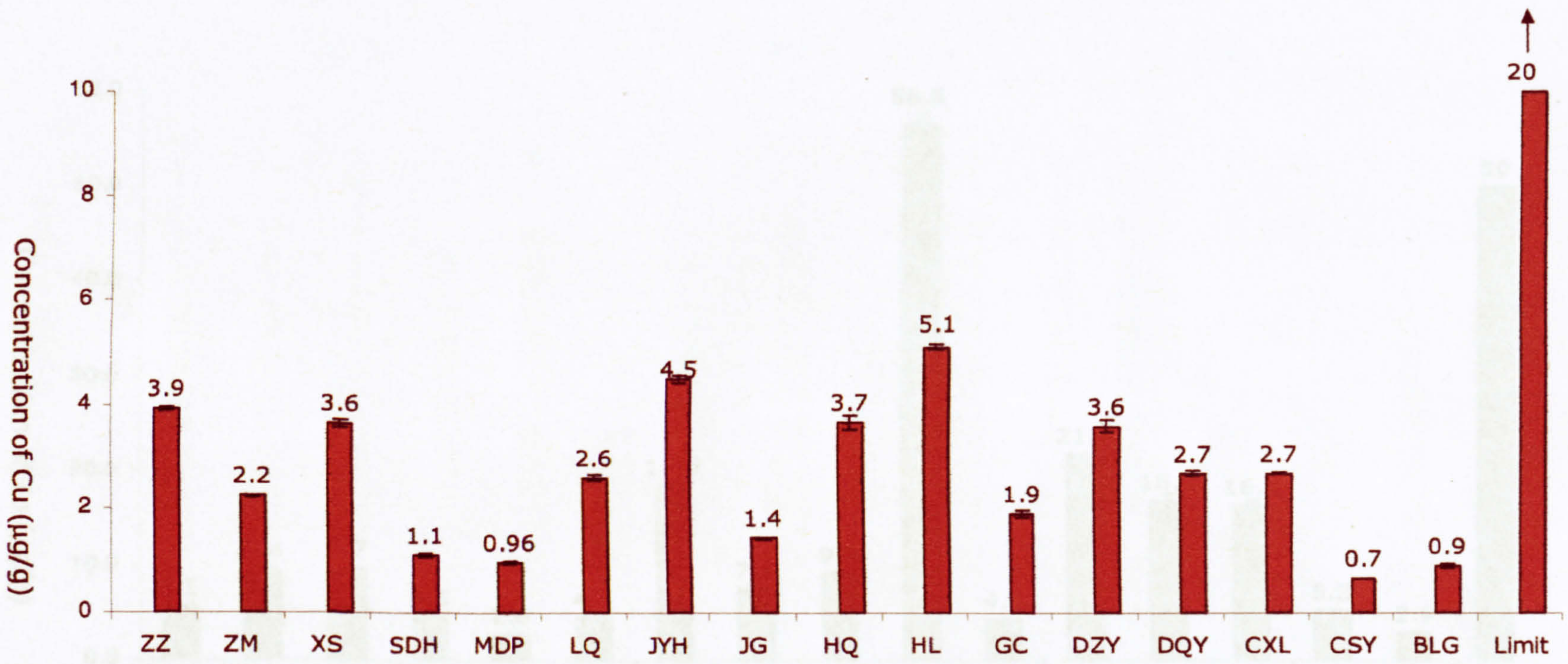


Fig. 3.7. Average concentrations of Cu in 16 herbs together with standard deviations (± SD).
The LOD for Cu in this study was 0.07 μg/g. In the UK, a limit of 20 μg/g of Cu in food was recommended by the Food Standards Committee (MAFF, 1956).

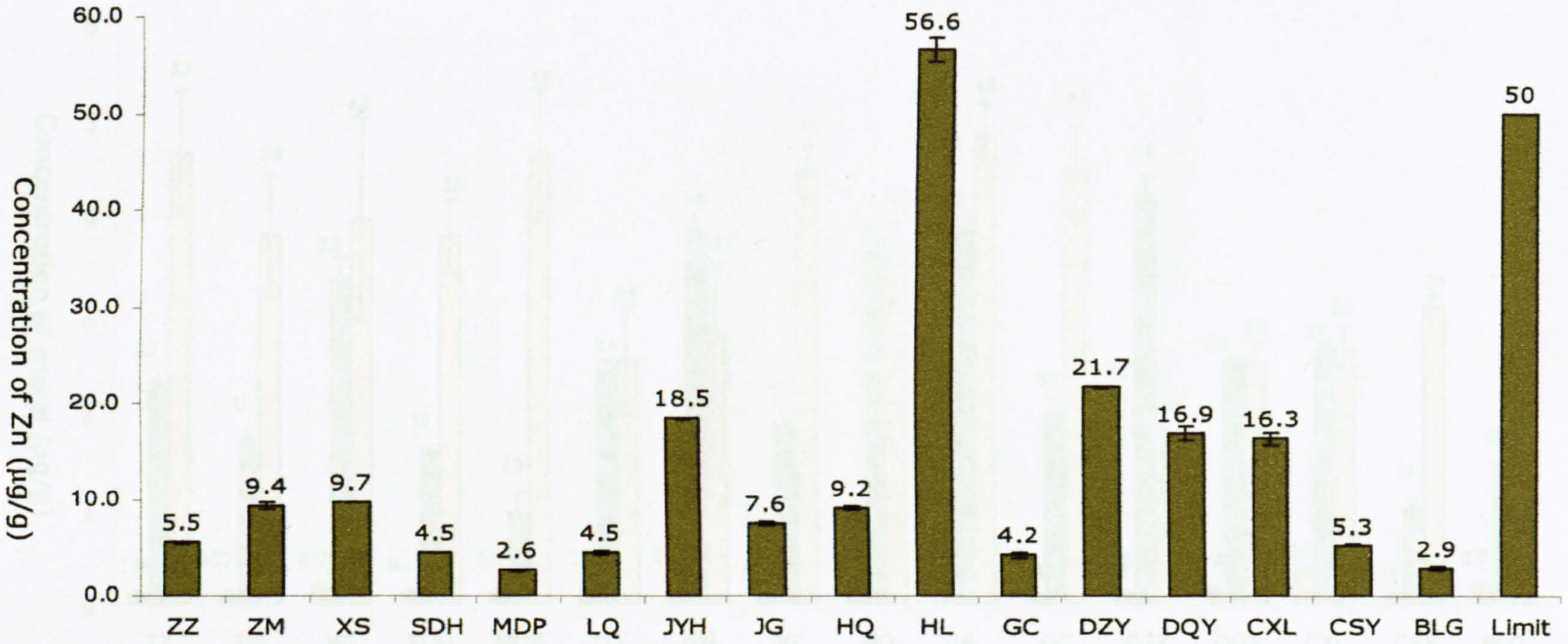


Fig. 3.8. Average concentrations of Zn in 16 herbs together with standard deviations (± SD).
The LOD for Zn in this study was 0.11 µg/g. In the UK, the general recommended limit of Zn in food is 50 µg/g (MAFF, 1956; Chuan *et al.*, 2000).

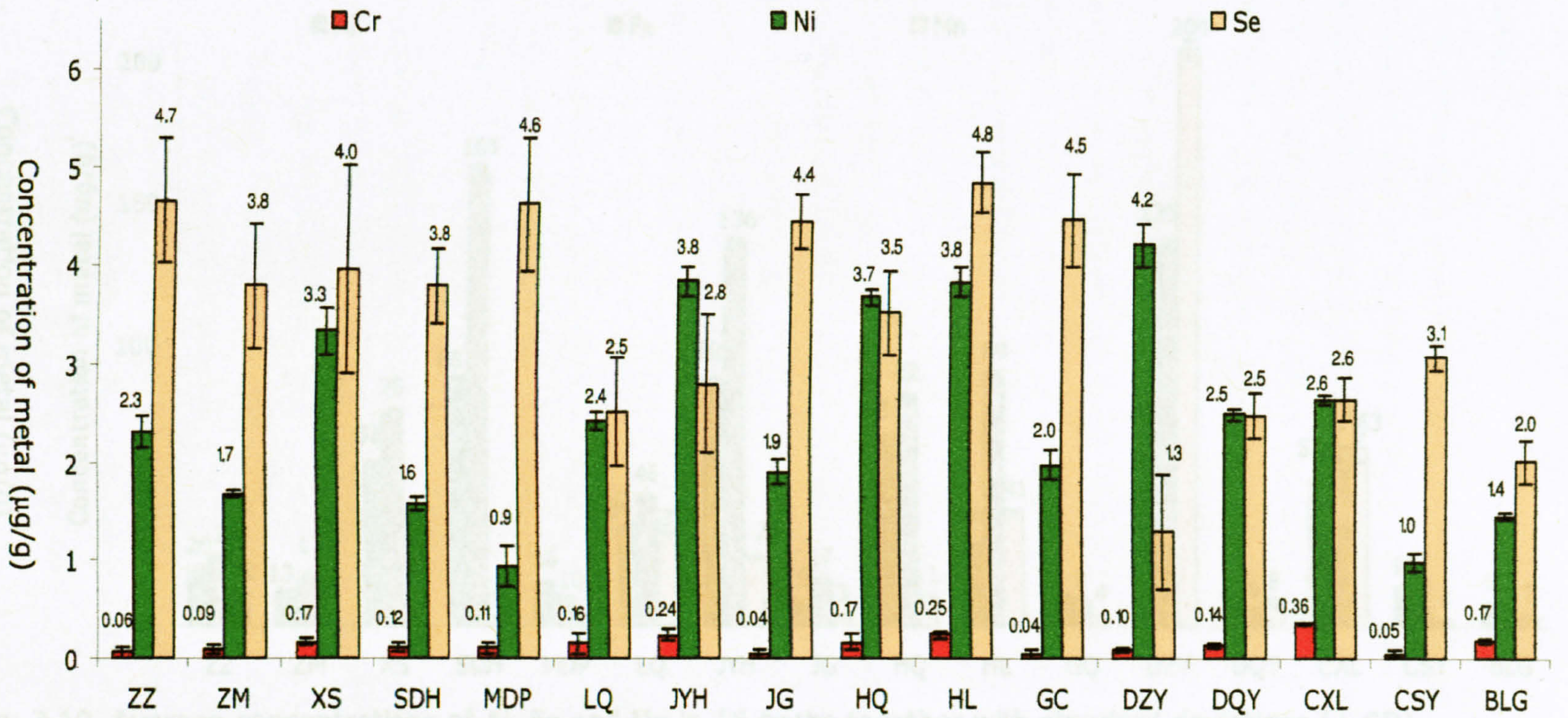


Fig. 3.9. Average concentrations of Cr, Ni and Se in 16 herbs together with standard deviations (± SD).
The LODs for Cr, Ni and Se in this study were 0.027 μg/g, 0.077 μg/g and 0.063 μg/g, respectively.

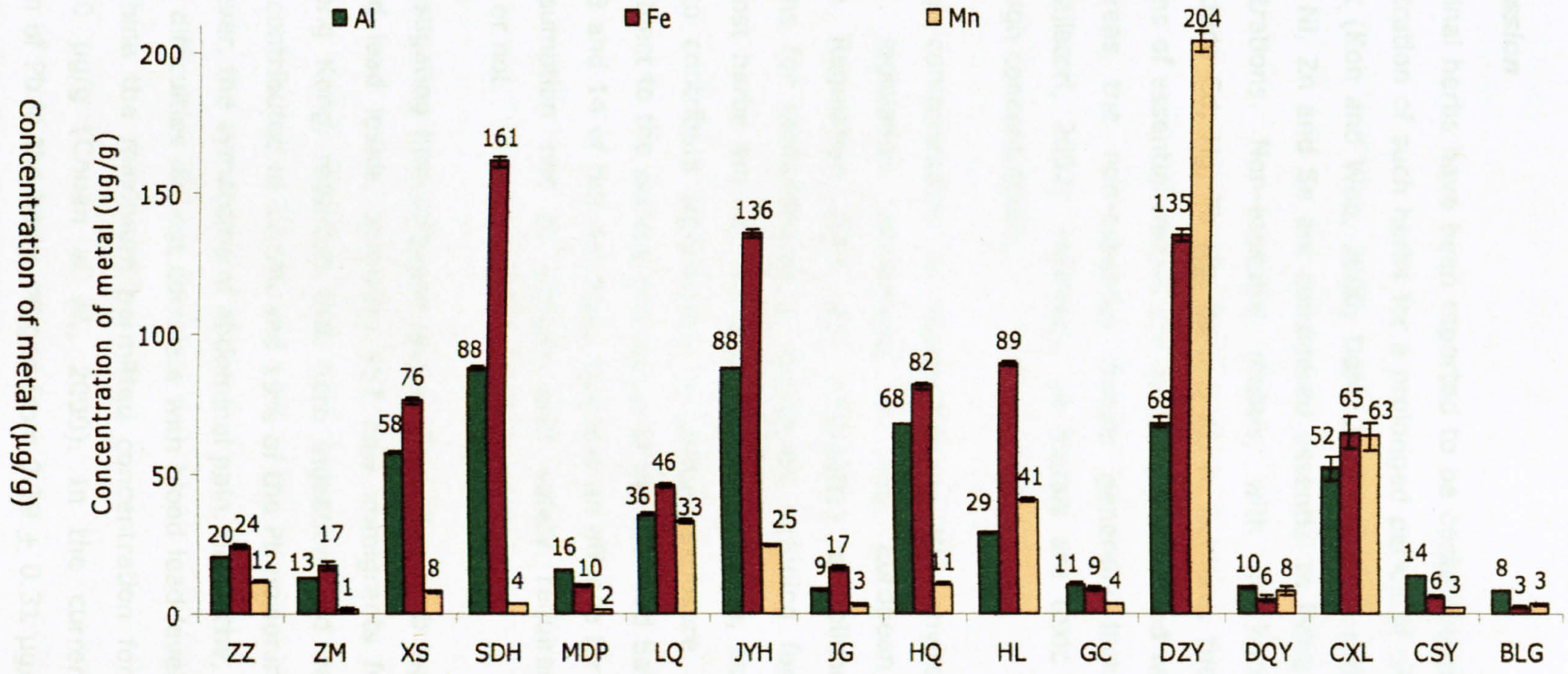


Fig. 3.10. Average concentrations of Al, Fe and Mn in 16 herbs together with standard deviations (± SD).
The LODs for Al, Fe and Mn in this study were 0.058 μg/g, 0.123 μg/g and 0.029 μg/g, respectively.

3.3.3 Discussion

Some medicinal herbs have been reported to be contaminated with metals and administration of such herbs for a prolonged period of time could pose a health risk (Koh and Woo, 2000; Dahl, 2001). Certain metals, including Cu, Fe, Mn, Ni, Zn and Se are considered essential to living organisms at low concentrations. Non-essential metals, with no known biological functions include Cd, Hg, Pb Hg, Be and Al. In humans, the "intracellular concentrations of essential metals are normally maintained within a narrow range, whereas the non-essential metals generally lack homeostatic controls" (Ballatori, 2002). However, all metals are toxic if present at sufficiently high concentrations.

Heavy metal contamination is widespread in the environment. Food contaminant legislation introduced in the European Community (Commission Regulation (EC) No. 466/2001) establishes maximum concentrations for contaminants in foodstuffs including for Pb and Cd. However, most herbs are not included in this legislation, as they are not considered to contribute significantly to dietary exposure. Nevertheless, herbs are subject to the general provisions of the UK Food Safety Act 1990. Sections 7, 8 and 14 of this Act state that it is an offence for food used for human consumption not to comply with safety requirements, be it intentionally or not.

A study investigating how different factors (including herb ingestion) affect venous blood lead levels, involving 457 new immigrants from mainland China to Hong Kong, reported that herb ingestion and drinking water, respectively contributed to 17.5% and 19% of the Pb poisoning (Ling *et al.*, 2002). However, the symptoms of abdominal pain, headache, short stature, and learning difficulties did not correlate with blood lead levels (Ling *et al.*, 2002). In China the maximum permitted concentration for Pb (in dried herbs) is 10 µg/g (Chuan *et al.*, 2000). In the current study, the concentration of Pb in the herbs ranged from 0.39 $\pm$ 0.31 µg/g (in the root of *Glycyrrhiza uralensis*, GC) to 2.27 $\pm$ 0.15 µg/g (in the aerial part of *Lophatherum gracile*, DZY) (Fig. 3.4). These values were well below the

legal limit of 10 µg/g. A concentration 25 ± 3.0 µg/g for Pb was measured in rhino horn extract, which was more than double the legal limit (Table 3.7).

There is no UK guideline limit for Cd and so the guideline limits of the country of origin of the food are referred to. In China, the current maximum permitted concentration for Cd in food is 0.3 µg/g (Chuan *et al.*, 2000). The concentrations of Cd were below the LOD (0.03 µg/g) in eight herbs namely, *Gardenia jasminoides* (ZZ), *Anemarrhena asphodeloides* (ZM), *Paeonia suffruticosa* (MDP), *Paeonia lactiflora* (CSY), *Platycodon grandiflorum* (JG), *Scutellaria baicalensis* (HQ), *Glycyrrhiza uralensis*, (GC), Isatidis Folium (DQY) and Isatidis Radix (ban lan gen, BLG) (Fig. 3.5). The highest concentration of Cd (0.12 ± 0.05 µg/g) was detected in *Lophatherum gracile* (DZY), which was still below the guideline limit for Cd (0.3 µg/g) in China (Fig. 3.5). However, a Cd concentration of 0.6 ± 0.13 µg/g was detected in rhino horn, which was more than twice the legal limit (Table 3.7).

The UK statutory limit for Sn in food, as set by the Food Regulations (1992), is 200 µg/g. In this study, the range of concentrations of Sn in the herbs ranged from 3.1 ± 0.10 µg/g and 3.1 ± 0.08 µg/g (in *Andrographis paniculata*, CXL and *Lophatherum gracile*, DZY, respectively) to 9.7 ± 0.21 µg/g (in *Paeonia suffruticosa*, MDP), (Fig. 3.6) and in rhino horn a value of 8.3 ± 1.2 µg/g was determined (Table 3.7). Therefore, these concentrations were well below the statutory limit.

In Singapore, the maximum legal concentration for Cu in tea is 150 µg/g (Chuan *et al.*, 2000). In the UK, a guideline limit of 20 µg/g of Cu in food was recommended by the Food Standards Committee, with specific limits for certain foods (MAFF, 1956). The concentrations of Cu in the herbs ranged from 0.7 ± 0.006 µg/g (in *Paeonia lactiflora*, CSY) to 5.12 ± 0.06 µg/g (in Coptis Rhizoma, HL) (Fig. 3.7). A Cu concentration of 4 ± 0.11 µg/g was found in rhino horn (Table 3.7). Therefore, all determined values for Cu were well below the recommended limit.

The concentration of Zn measured in the herb Coptis Rhizoma, (HL) was 56.6 ± 1.3 µg/g (Fig. 3.8) and in rhino horn the value was 69.8 ± 0.08 µg/g (Table 3.7). Both these values were higher than the general guideline limit of 50 µg/g of Zn in food (MAFF, 1956; Chuan *et al.*, 2000). The range of Zn concentrations in the rest of the tested herbs ranged from 2.6 ± 0.05 µg/g (in *Paeonia suffruticosa*, MDP) to 21.7 ± 0.15 µg/g (in *Lophatherum gracile*, DZY) (Fig. 3.8).

Ag, Be, Co and Hg were not detected below the LODs (0.25, 0.07, 0.07 and 0.17 µg/g, respectively) in all the herbs. In rhino horn, Ag and V were the only two metals not detected (above their LODs of 0.15 µg/g and 0.29 µg/g, respectively (Table 3.7)). A concentration of Hg of 2.3 ± 1.4 µg/g was detected in rhino horn, which demonstrated consistent exceedence of the legally permissible limit of Hg (0.5 µg/g) in Singapore (Chuan *et al.*, 2000).

Currently, there are no available legal limits for Cr, Ni, Se (results shown in Fig. 3.9) and Al, Fe and Mn (results shown in Fig. 3.10). Compared to the other metals tested in this study, high levels of Al, Fe and Mn (Fig. 3.10) were detected in several of the plant materials. In the herbs, the highest measured concentrations of Al and Fe were 88.2 ± 0.81 µg/g and 161 ± 2.2 µg/g, respectively in the root of *Rehmannia glutinosa* (SDH). The highest concentration of Mn (204 ± 3.6 µg/g was found in the aerial part of *Lophatherum gracile* (DZY). Rhino horn contained even higher concentrations of Al (236 ± 5.0 µg/g) and Fe (321 ± 9.7 µg/g) but a comparatively lower concentration of Mn (7.6 ± 0.08 µg/g).

The plant materials and rhino horn used in this study were not washed before analysis in order to simulate the procedures during TCM use, when it is not usual to wash the herbs prior to prescription preparation. Several of the metals analysed (particularly Al, Fe and Mn) are resident in soil as well as pollutants in the atmosphere. Due to the tendency for some metals to accumulate in plant material, food crops, herbs and tree barks are sometimes used as biomonitors of the metal pollution in different environments (Ward and Savage 1994; Murphy *et al.*, 2000; Narewski *et al.*, 2000). Ward and Savage (1994) conducted multielemental analyses of

soil, washed and unwashed food exposed to different motor vehicle activities. Out of the metals analysed (including Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn), only Pb had a concentration higher for unwashed plant material and surface soils than washed plant material (Ward and Savage 1994). They also reported that 40-80% of Pb could be removed by washing plant surfaces (Ward and Savage, 1994). The process of preparing herbs, by boiling in water, for consumption as a medicine or tea may affect the availability of some metals. In a study conducted in Hungary, 23 elements were determined in the herb *Silybum marianum* L. Gaertner (Szentmihalyi *et al.*, 1998). Significant levels of some elements were observed and it was found that when the herb was made into a tea the concentrations of some of the residual metals decreased (Szentmihalyi *et al.*, 1998).

The results from this study do not give a clear indication as to whether herbs prepared using a particular part of the plant affect its metal content. In a study conducted in China, ICP was used to compare the concentrations of metals (including Cu, Fe, Mn, and Zn) in soil and in the rhizomes and leaves of *Rheum palmatum* and *R. likiangense* plants (Xie, 2000). It was reported that there was no significant difference between Fe in leaves and rhizomes but that soil concentrations were 50-100 times higher. Mn and Cu levels were highest in soil, followed by leaves and then rhizomes (Xie, 2000). Also, in general, there was not a significant difference between the metal concentrations in the different plant species (Xie, 2000). In a study conducted by Murphy et al. (2000) to monitor the uptake of different metals by plants, it was shown that "Zn and Cu exhibited the greatest potential to migrate from roots to the leaves", with Pb being retained principally in the roots of the vegetation.

Preliminary results from this study indicate that the rhino horn sample contained unacceptable concentrations of toxic metals such as Cd, Pb and Hg as well as Zn. To the authors knowledge this is the first report on metal analysis of rhino horn in the English language. Rhinos are herbivores and a factor which can influence the metal content of rhino samples is diet. Another important consideration is the storage conditions of rhino horn samples which could contribute to the accumulation of metals over a period

of time. All the sixteen herbs tested complied with the current maximum permissible concentrations established for Cd, Pb and Sn. Hg was not detected in the herbs above the LOD of the method. Only one herb contained a Zn concentration which was above the legal limit.

3.4. Quantitative Analysis of Pesticide Residues

The analytical method used conforms to the United Kingdom Accreditation Service (UKAS) accreditation certificate for the determination of pesticide residue levels in fruits and vegetables. The analyses were conducted at the TDL Section of Leatherhead International Ltd, Surrey.

3.4.1. Experimental procedure

Pesticide residue analyses were performed using gas chromatography (GC) equipped with both a nitrogen phosphorus detector (NPD) and an electron capture detector (ECD). Volatile organic compounds containing phosphorus and/or nitrogen moieties were detected with the NPD detector, whilst those with chlorine moieties were detected with the ECD detector. Mixed standards of pesticides were analysed and their relative retention times (RRT) to the internal standards were calculated. The RRTs of peaks in test samples were then compared to those of the standards. Confirmatory analyses of pesticides detected in the herbal extracts were performed using a second chromatographic column with a different polarity as stated by the Commission Recommendation 1999/333/EC (section 63). Pesticide residues confirmed by the second GC were analysed using GC-mass spectrometry (GC-MS) with the corresponding pesticide standard as a reference.

3.4.1.1. Mixed calibration standards

Fourteen herbs (Table 3.4) were analysed for 125 pesticide residues. The organophosphorus (OP) and organonitrogen (ON) pesticides were divided

into six mixed standards (OPON A to OPON F; Table 3.8). The organochlorine (OC) standards were divided into four mixed standard solutions (OC A to OC D; Table 3.9). For the purpose of spiking a sample to calculate the recoveries of pesticides (represented by a range of elution times), separate mixtures of OPON MIX and OC MIX standards were prepared (Table 3.10). A mixed internal standard solution was prepared containing isodrin (5 µg/ml; internal standard for OC pesticides) and triphenyl phosphate (TPP, 7.5 µg/ml; an internal standard for OP and ON pesticides). All standards were prepared using ethyl acetate as the solvent.

3.4.1.2. Extraction

Dried herbs (5 g) containing a mixed internal standard (1 ml) were extracted using ethyl acetate (50 ml). In order to detect lower concentrations of pesticide residues, 10 ml aliquots of the herbal extracts were dried down under a stream of nitrogen gas and the dried extract was re-dissolved in 1 ml ethyl acetate to produce a ten times more concentrated extract (1 mg/ml) for GC analysis. A sample of *Lonicera japonica* was spiked with OPON MIX standard solution and *Gardenia jasminoides* was spiked with OC MIX standard solution.

3.4.1.3. GC and GC-MS methods

The GC system used to perform the two separate analyses (using columns with different polarities for confirmation of compounds detected) was a Perkin-Elmer AutoXL. A first chromatographic separation was performed using a 30 m x 0.25 mm i.d. x 0.25 µm DB5 capillary column. The initial oven temperature was 60°C with an initial hold time of 2 minutes followed by an increase from 60-180°C at 20°C/min and then a second ramp rate of 6°C/min from 180-300°C, with a final hold time of 14 minutes. The carrier gas was helium. Injections of 4 µl in a split injection mode at 280°C were made by an autosystem. Detection was by both NPD and ECD.

For the second chromatographic separation, a DB210 capillary column was used and the second ramp rate of 6°C/min was from 180-280°C, with a subsequent hold time of 13 minutes. All other conditions were the same as for the first GC analysis.

Further confirmatory analyses were performed on a GC-MS system consisting of a Hewlett Packard GC 5890 coupled to a Hewlett Packard MSD quadrupole analyser. Chromatography was performed on a 30 m x 0.25 mm i.d. x 0.25 µm WCOT fused silica capillary column with a CP-SIL 8 CB coating (DB1701). The initial temperature of 60°C was held for 1 minute followed by a 20°C/min rise to 180°C and then 180-300°C at 6°C/min with no hold time. The carrier gas was helium and manual injections of 2 µl (splitless) at 280°C were made.

3.4.2. Results

From the chromatograms obtained from GC analyses, the relative retention times (RRT) of pesticides contained within the internal standards were calculated (Tables 3.8 and 3.9). The RRT of the peaks obtained for the herbal extracts were compared to those of the standards, subject to an acceptable difference of $\pm$ 0.005 minutes. As part of the quality assessment of the method, the RRT of peaks from the blank (ethyl acetate) analysis were also compared to those of the standards. There were no peaks from the blank analysis corresponding to any pesticide residues.

The recoveries (Table 3.10) obtained for 20 pesticides ranged between 78.7-115.6% for 0.1 g/ml samples. At 1 g/ml, comparatively higher recoveries of 85.2-283.6% were obtained, with a value of 283.6% for methoxychlor, which was obviously erroneous. Methoxychlor was detected in only one herbal extract (*Forsythia suspensa*) by GC (Table 3.11) but it was not confirmed by GC-MS and therefore, the GC analysis was not repeated. The range of acceptable recoveries, per batch, stated in the Commission Recommendation 1999/333/EC (section 56) is 75 – 110%.

Pesticide residues were identified as being present in all of the 14 herbs analysed (Table 3.11), which GC-MS analysis subsequently proved to be false positives. Results obtained from the analysis of the herb Isatidis Radix have been used as an illustration of the interpretation of the chromatograms (Figs. 3.11-3.13).

Table 3.8. Details of the six organophosphorus/organonitrogen (OPON) pesticide mixed standards including their limits of detection (LOD) and relative retention times (RRT) of the individual pesticides using GC-NPD.

Pesticides in OPON A	LOD, µg/g	RRT	Pesticides in OPON B	LOD, µg/g	RRT
azinphos ethyl	0.05	1.154	Dioxathion	0.05	1.207
profenphos	0.05	0.850	azinphos methyl	0.05	1.107
quinalphos	0.01	0.784	Ethion	0.01	0.918, 0.368
mercarbam	0.01	0.776	Iodofenphos	0.05	0.842
bromophos-methyl	0.01	0.746	bromophos ethyl	0.01	0.803
fenthion	0.05	0.911	Isofenphos	0.02	0.772
pirimiphos-methyl	0.01	0.688	Chlorpyrifos	0.01	0.716
parathion-methyl	0.02	0.661	Fenitrothion	0.02	0.695
formothion	0.05	0.617	tolclofos methyl	0.01	0.664
diazinon	0.01	0.590	Phosphamidon	0.10	0.642
atrazine	0.05	0.568	Dioxathion	0.05	0.368
phorate	0.01	0.536	Thiometon	0.01	0.313, 0.297
ethoprophos	0.05	0.502	Omethoate	0.01	0.486
mevinphos	0.01	0.400	Heptenophos	0.01	0.470
trichlorfon	0.01	0.323	Disulfoton	0.01	0.358
			Dichlorvos	0.01	0.325
			Oxamyl	0.01	0.257
pyrazophos	0.02	1.140	Fenbuconazole	0.05	1.236
carbofenthion	0.01	0.957	Prochlorax	0.10	1.207
triazophos	0.02	0.941	Bitertanol	0.05	1.182
fenamiphos	0.02	0.832	Iprodione	0.50	1.033
Methidathion	0.02	0.807	Propiconazole	0.05	0.971
Chlorvenvinphos	0.01	0.775	fluazifop butyl	0.10	0.886
Vamidothion	0.10	0.759	Tetrachlorvinphos	0.05	0.814
parathion ethyl	0.01	0.725	Pendamethalin	0.05	0.761
Malathion	0.01	0.704	Diethofencarb	0.20	0.711
Fenchlorphos	0.01	0.676	Carbofuran	0.05	0.633

Table 3.8 cont'd.

Pesticides in OPON C	LOD, µg/g	RRT	Pesticides in OPON D	LOD, µg/g	RRT
chlorpyrifos methyl	0.01	0.655	propoxur	0.05	0.562
Etrimphos	0.01	0.611			
Fonophos	0.02	0.592			
Dimethoate	0.05	0.561			
Methacriphos	0.01	0.432			
Methidathion	0.02	0.324			
Phosalone	0.01	1.099	Benalaxyl	0.05	0.954
Phosmet	0.05	1.046	Buprimate	0.05	0.866
Oxadixyl	0.10	0.920	Hexaconazole	0.05	0.843
Cyproconazole	0.05	0.890	disulfoton-sulfone	0.20	0.820, 0.368
Myclobutanil	0.05	0.864	Cyprodinil	0.05	0.761
Paclobutrazole	0.05	0.815	Triadimefon	0.02	0.727
Penconazole	0.05	0.769	Metalaxyl	0.05	0.671
phorate sulfone	0.05	0.717	Pyrimethanil	0.05	0.597
Pirimicarb	0.05	0.624	Ethoxyquin	0.20	0.558
Diphenylamine	0.05	0.501	demeton-S-sulfone	0.05	0.497
3,4-dichloroaniline DCA)		0.394	Methamidophos	0.02	0.343

The LOD for each pesticide standard analysed was calculated from the standard deviation obtained from analysis of a blank sample (ethyl acetate).

Table 3.9. Details of the four organochlorine pesticide mixed standards including their limits of detection (LOD) and relative retention times (RRT) of the individual pesticides using GC-ECD.

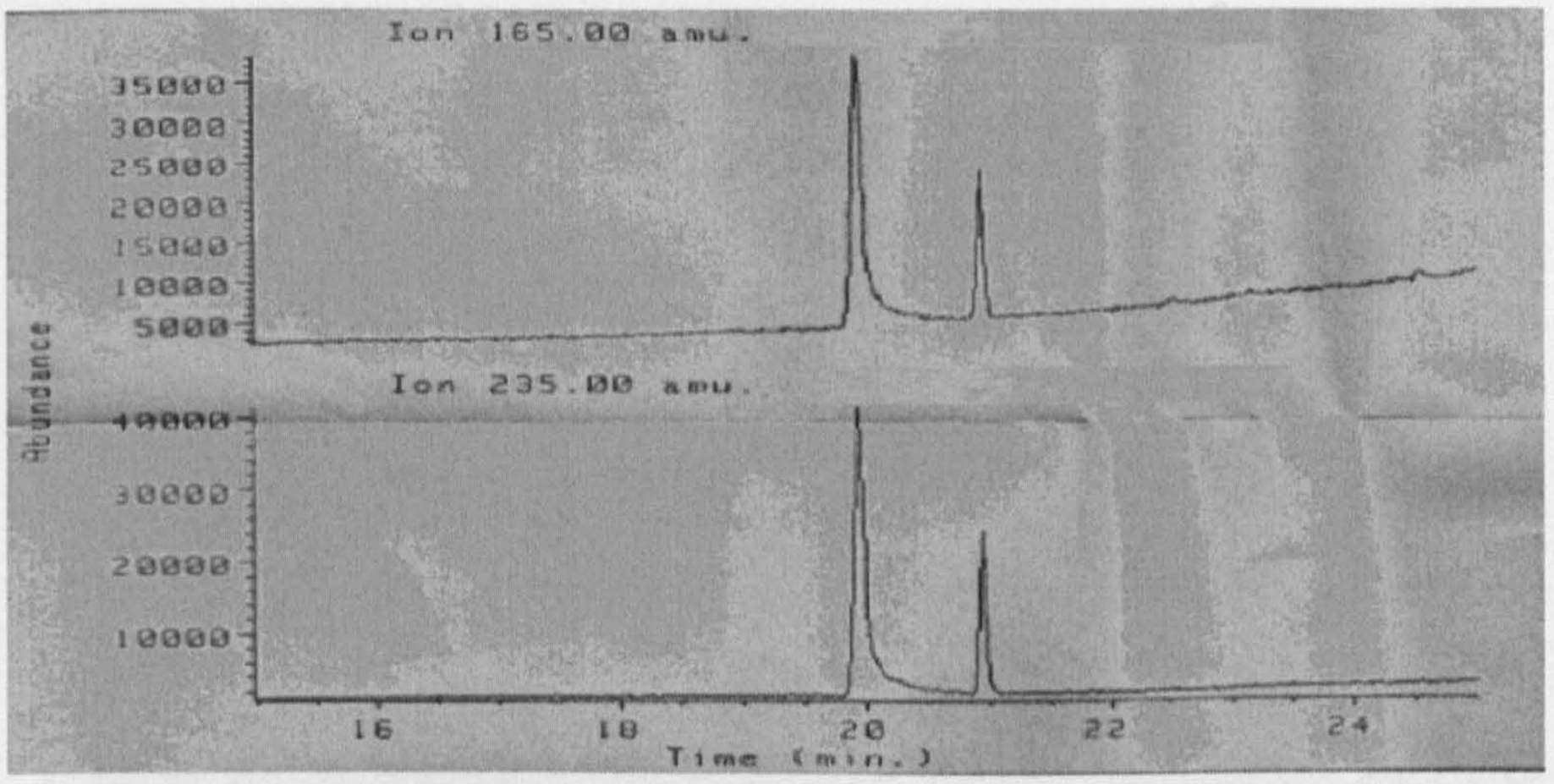
Pesticides in OC A	LOD, µg/g	RRT	Pesticides in OC B	LOD, µg/g	RRT
hexachlorobenzene	0.02	0.726	Propachlor	0.03	0.644
Lindane	0.01	0.769	Quintozene	0.01	0.764
Heptachlor	0.01	0.884	Vinclozin	0.01	0.859
Aldrin	0.01	0.946	Heptachlor	0.01	0.984
heptachlor epoxide	0.02	1.016	heptachlor epoxide	0.02	1.023
Folpet	0.03	1.048	trans-chlordane	0.01	1.060
op-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene)	0.01	1.062	alpha-endosulfan	0.01	1.086
cis-chlordane	0.01	1.086	Dieldrin	0.01	1.136
op-TDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane)	0.01	1.138	beta-endosulfan	0.01	1.200
Endrin	0.01	1.180	endosulfan sulphate	0.02	1.274
pp-TDE	0.01	1.204	Bifenthrin	0.05	1.360
pp-DDT (dichlorodiphenyltrichloroethane)	0.01	1.276	Cyhalothrin	0.05	1.446, 1.465
Bromopropylate, endosulfan sulphate	0.01	1.372	Permethrin	0.05	1.541, 1.554, 1.567
Tetradifon	0.01	1.430	Cypermethrin	0.05	1.649, 1.659 1.666, 1.670
Fenamirol	0.01	1.499			
Cyfluthrin	0.05	1.615, 1.625, 1.632, 1.636			
Deltametrin	0.05	1.706, 1.808, 1.820, 1.847			
Trifluralin	0.01	0.670	Pentachlorobenzene, (PCB)	0.01	0.592
α-HCH (Hexachlorocyclohexane)	0.01	0.718	Tecnazene	0.01	0.640
β-HCH	0.01	0.758	Dichloran	0.01	0.738
Dicofol	0.02	0.963	Propyzamide	0.02	0.769
Tolyfluanid	0.03	1.014	Chlorthalonil	0.02	0.803
Procimydone	0.02	1.035	Dichlorfluanid	0.02	0.924
pp-DDE	0.01	1.121	Captan	0.02	1.036
kresoxim-methyl	0.01	1.135	Chlorfenson	0.01	1.104
op-DDT	0.01	1.209	Chlorbenzilate	0.02	1.182
Fenpropathrin	0.05	1.382	Methoxychlor	0.01	1.383
Flucythrinate	0.05	1.577, 1.667, 1.688	Fenvalerate	0.01	1.661, 1.750, 1.775
Azoxystrobin	0.02	1.879			

Table 3.10. The percentage recovery of 20 pesticides as calculated from GC-ECD and GC-NPD analysis

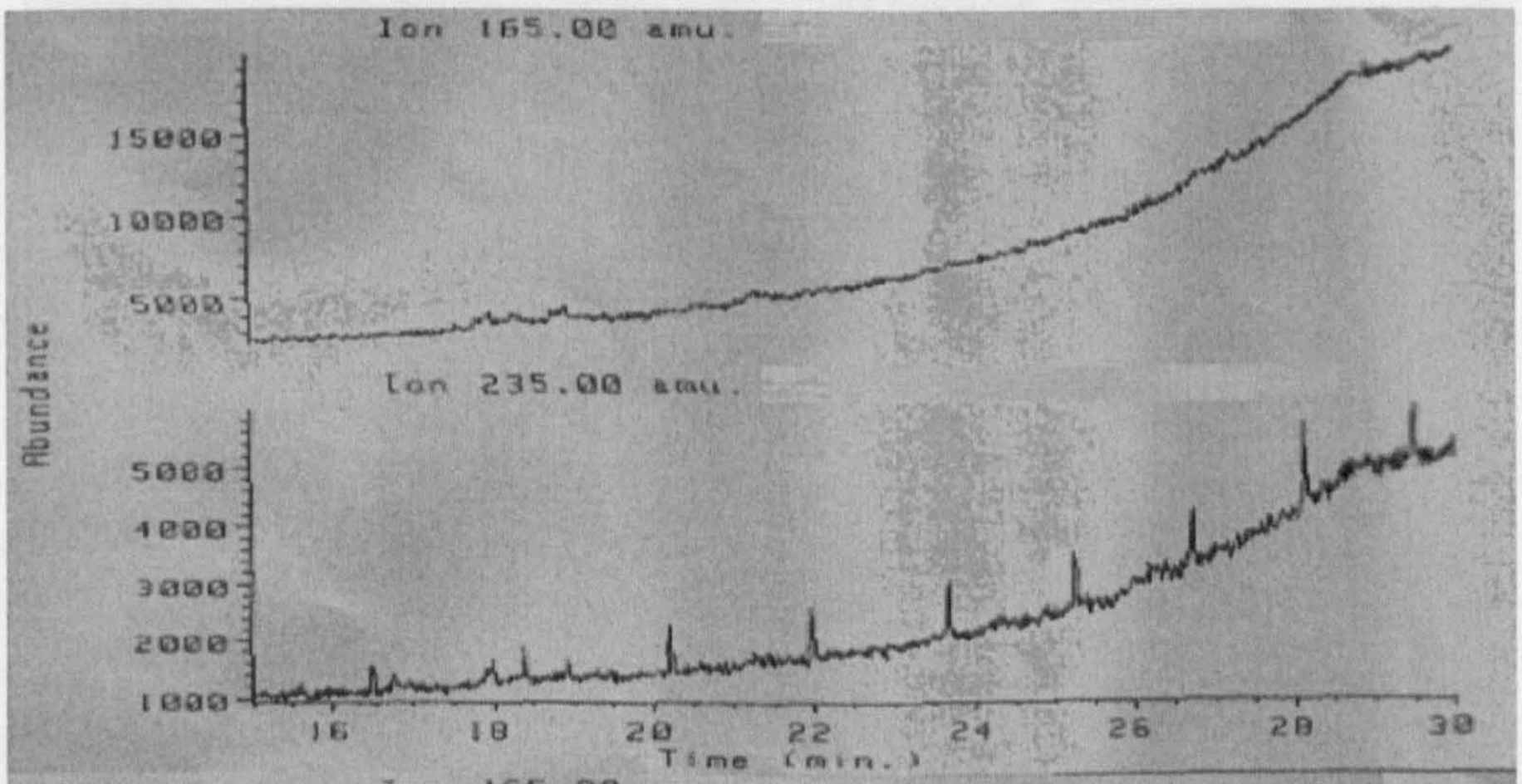
Pesticide	% Recovery (0.1 g/ml samples)	% Recovery (1 g/ml samples)
OC MIX (analysed using GC-ECD)		
dieldrin	105.1	133.2
pp-DDE	104.7	139.4
aldrin	101.6	136.0
tecnazene	99.4	98.4
α-endosulfan	98.4	110.3
methoxychlor	98.4	283.6
heptachlor	85.8	94.5
op-DDT	78.8	198.8
OPON MIX (analysed using GC-NPD)		
triadimefon	115.6	114.2
prochloraz	101.3	87.6
pirimicarb	93.8	131.8
carbofenthion	91.8	115.3
fluazifop-butyl	89.3	95.6
buprimate	88.8	89.8
chlorpyriphos	88.3	116.5
pirimiphos methyl	86.7	93.7
penconazole	86.5	98.3
ethion	84.6	103.4
methacriphos	84.4	85.2
diazinon	97.3	93.1

Lonicera japonica was spiked with OPON MIX standard solution and, *Gardenia jasminoides* was spiked with OC MIX standard solution. At 1 g/ml, recoveries of 283.6% and 198.8% were obtained for methoxychlor and op-DDT, respectively when analysed with GC-ECD. These errors could have occurred if these pesticides were co-eluting with other compounds leading to elevated sample response, and subsequently erroneously high recovery efficiency. Since the identification of the peaks was entirely based on retention time, this assumption was not verified. However, results obtained from subsequent analyses of other herbal extracts using GC-ECD, GC-NPD (Table 3.11) were proved to be false negatives when the extracts were re-analysed using GC-MS. The GC-ECD recovery analysis was not repeated since the presence of pesticide residues in the extracts were not confirmed by GC-MS.

A



B



C

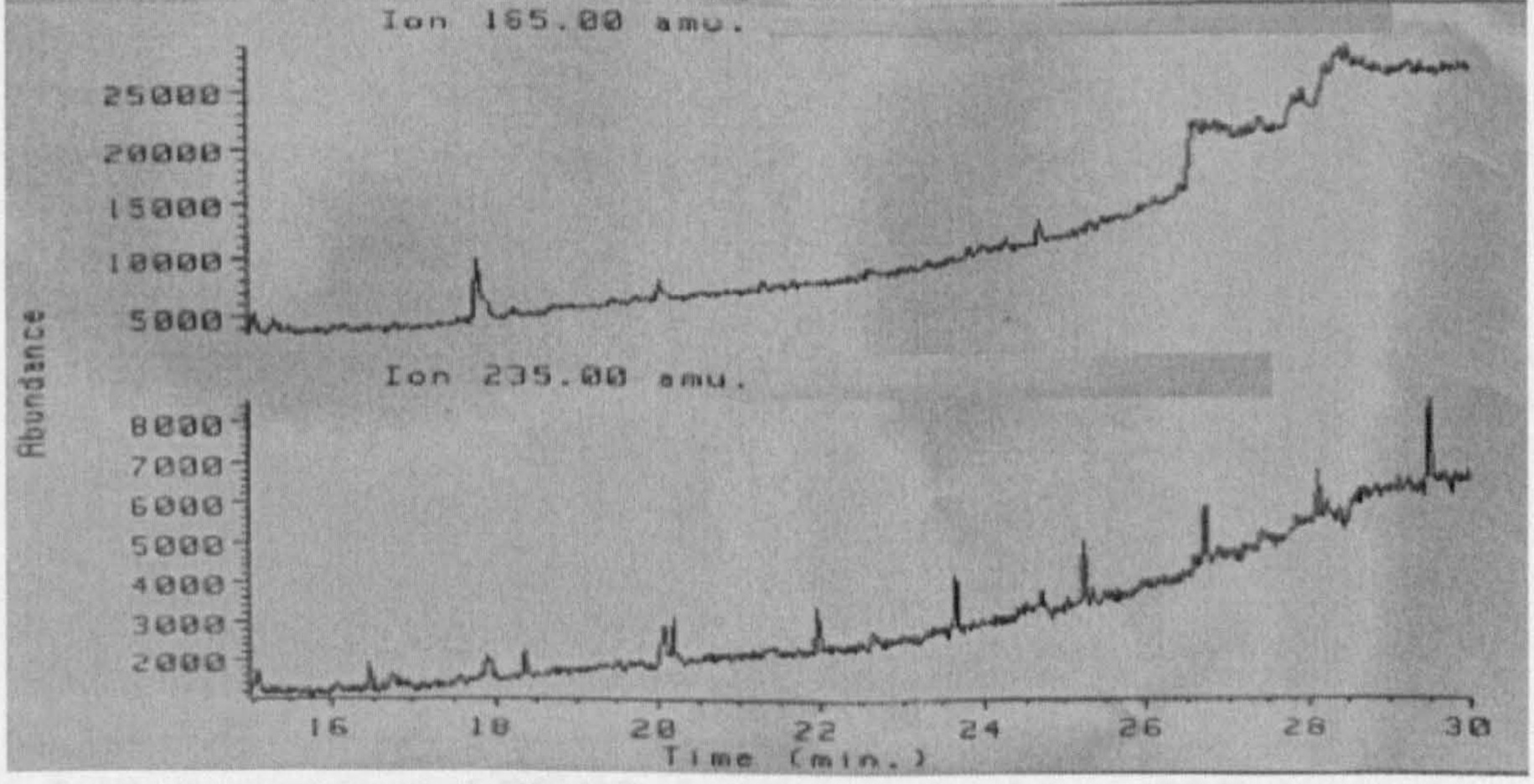


Fig. 3.11. Comparison of GC-MS chromatograms for op-DDT standard (A), Isatidis Radix (B) and *Lonicera japonica* (C) using specific ion monitoring at 165 amu and 235 amu.

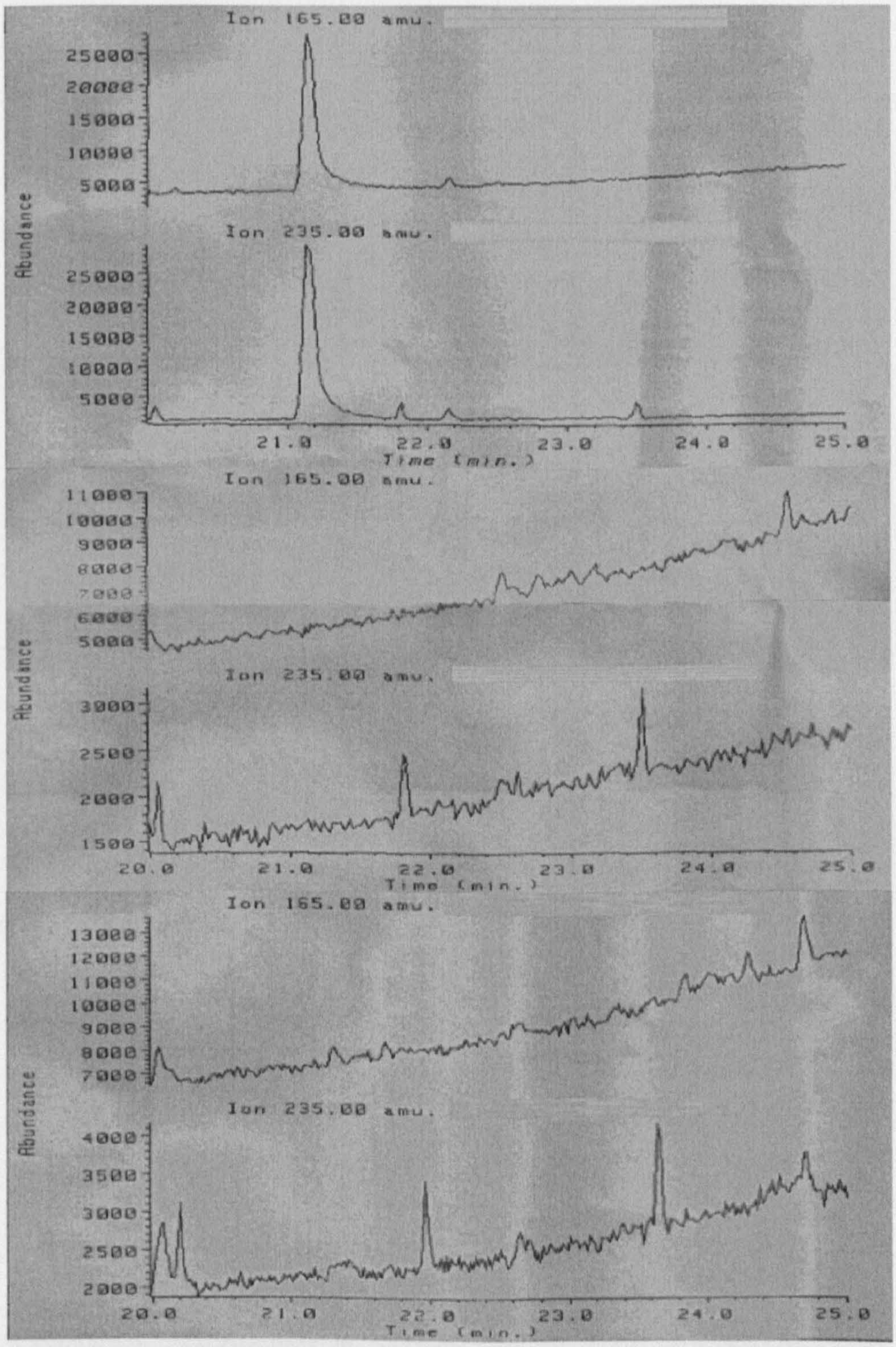
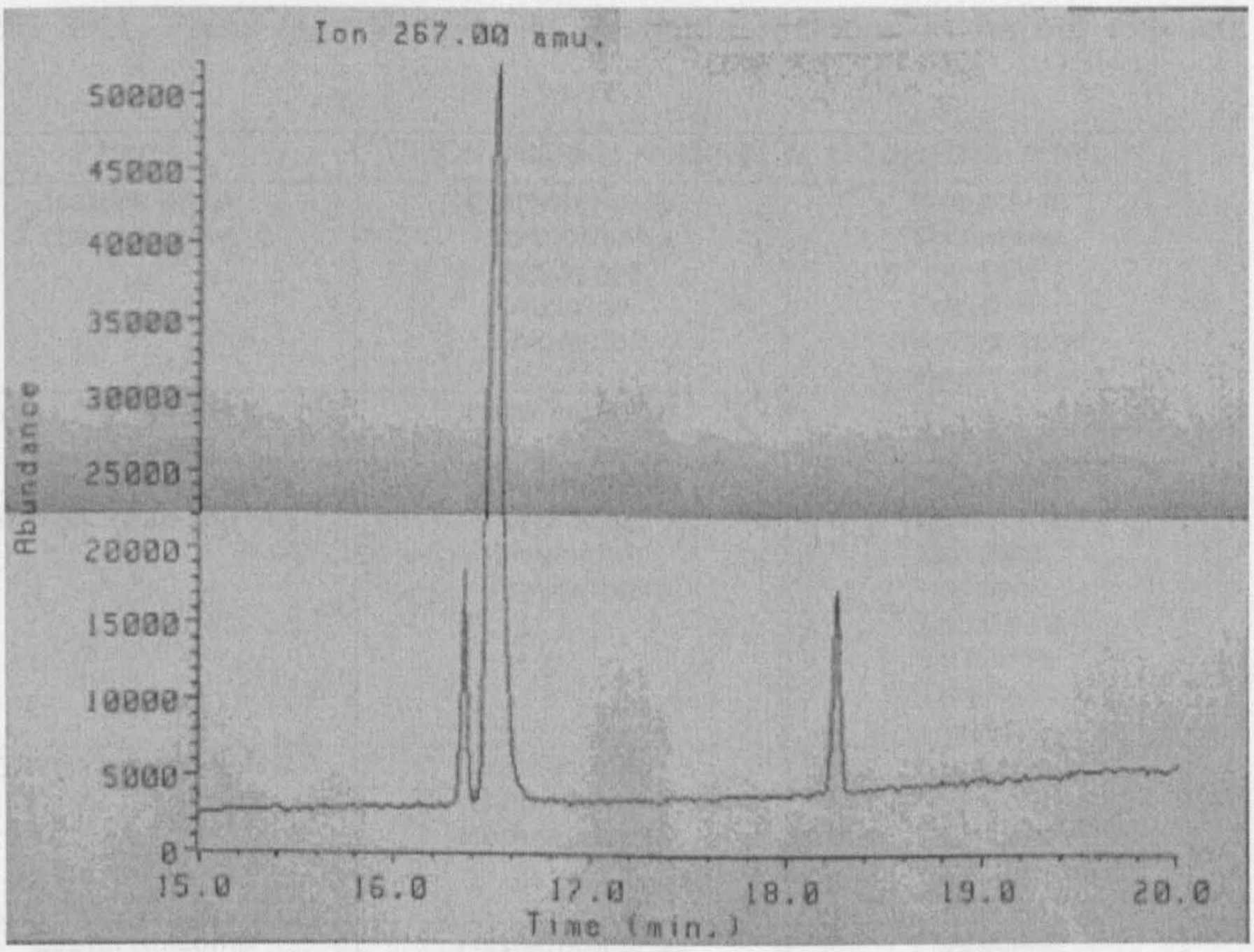


Fig. 3.12. Comparison of GC-MS chromatograms for pp-DDT standard (A) *Rehmannia glutinosa* (B) and *Lonicera japonica* (C) using specific ion monitoring at 165 amu and 235 amu.

A



B

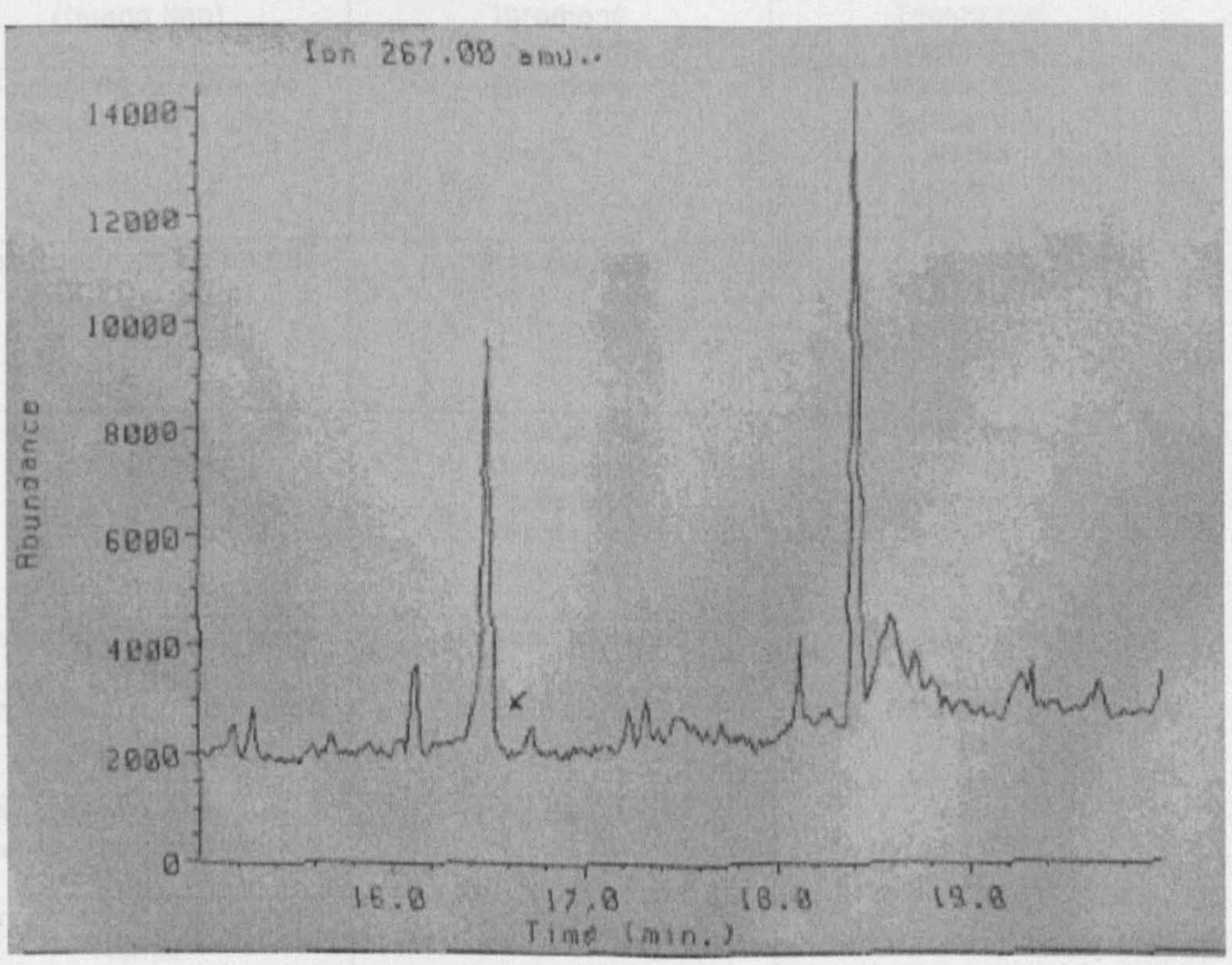


Fig. 3.13 Comparison of GC-MS chromatograms for diethofencarb standard (A) Isatidis Radix (B) using specific ion monitoring at 267 amu.

Table 3.11. False identification of pesticide residues in herbal extracts by GC.

Herbs	OP/ON pesticide residues	OC pesticide residues
Isatidis Radix (ban lan gen)	Diethofencarb Formothion Etrimphos Atrazine Thiometon	Propachlor Tecnazene op-DDE op-DDT Cis-chlordane Trans-chlordane
Paeonia lactiflora Pall. (chi shao yao)	none detected	Propachlor Lindane
Lophatherum gracile Brongn. (dan zhu ye)	Pendamethalin Cyprodinil Mevinphos Vamidothion	Propachlor Tecnazene pp-DDE Lindane Quintozene Dichloran Trifluralin Endrin
Glycyrrhiza uralensis Fisch. (gan cao)	Methacriphos Diethofencarb Methamidophos	Propachlor pp-DDE Dichloran Dicofol Aldrin
Coptis Rhizoma (huang lian)	Methamidophos Iprodione Penconazole	Propachlor Tecnazene Heptachlor
Scutellaria baicalensis Georgi (huang qin)	Iprodione	Propachlor Tecnazene Aldrin Endrin Quintozene
Platycodon grandiflorum A.DC. (jie geng)	none detected	Propachlor Lindane Dicloran Aldrin Endrin
Lonicera japonica Thunb. (jin yin hua)	Methidathion Dichlorvos Dioxathion Trichlorfon Mevinphos	Dichloran Dicofol op-DDT pp-DDT Fenvalerate Permethrin
Forsythia suspensa Vahl (lian qiao)	Pendamethalin	Methoxychlor
Paeonia suffruticosa Andr. (mu dan pi)	none detected	Propachlor Lindane Vinclozin
Rehmannia glutinosa Steud. (sheng di huang)	none detected	Aldrin pp-DDT Heptachlor epoxide
Scrophularia ningpoensis Hemsl. (xuan shen)	none detected	none detected
Anemarrhena asphodeloides Bge. (zhi mu)	Methacriphos	none detected
Gardenia jasminoides Ellis (zhi zi)	Methidathion	Chlorthalonil Flucythrinate

(Results were not confirmed by GC-MS).

3.4.3. Discussion

The European Community Council Directive (79/117/EEC) prohibits the commercial use of several persistent organochlorine compounds which are harmful to humans or the environment. Additionally, under Commission Directive 2000/81/EC, Maximum Residue Levels (MRLs) of pesticide residues in products of plant origin are set but no specific levels exist for medicinal herbs. In the current study, GC-ECD and GC-NPD analysis of herb extracts from 14 plant species indicated the presence of pesticide residues in all the extracts. Several of these suggested pesticides (aldrin, chlordane, dieldrin, dichlorodiphenyltrichloroethane (DDT), endrin, heptachlor, and quintozene and dicofol) are banned substances under the Council Directive 79/117/EEC. Further confirmatory analysis by GC-MS failed to confirm the presence of these pesticide residues in any of the herb extracts.

Some of the profiles of metabolites identified in the GC were indicative of false positives. For example, in determining whether the organochlorine, DDT had recently been applied to a sample, the detection of a combination of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (p,p-DDT) and 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene (p,p-DDE), the principal metabolite of DDT (Lackmann, 2005), by GC is more likely to yield a positive result when confirmed by GC-MS (or another identification method). In this study, although isomers and metabolites of DDT were detected by GC-ECD analysis of the herbal extracts (Table 3.11), for example, pp-DDT in *Rehmannia glutinosa* and *Lonicera japonica* and pp-DDE in *Lophatherum gracile* and *Glycyrrhiza uralensis*, the combination of p,p-DDT and p,p-DDE was not found in any of the samples. Consequently, results from GC-MS analysis showed that they were false positives.

The GC-MS profiles of extracts for different herbs of different species were sometimes similar when monitored at the specific ion masses. For example, *Lonicera japonica* and Isatidis Radix showed similar profile when monitored at 165 amu and 235 amu (Figs. 3.11B and 3.11C, respectively). Also, *Rehmannia glutinosa* and *Lonicera japonica* had similar profiles at 165 amu and 235 amu (Fig. 3.12B and 3.12C, respectively). However, the GC-MS profiles of the herb extracts at the specific ion monitoring did not match

those of the standards as shown in Figs. 3.11A (for op-DDT) and Figs. 3.12B (for pp-DDT). For some profiles there were only slight differences in retention times between the standard and the samples, which were considered significant due to the specificity of the technique. This is illustrated in Fig. 3.13 using diethofencarb standard and Isatidis Radix extact.

Secondary to its original purpose, the results from this study have further highlighted the unreliability of retention times for the chromatographic identification of specific compounds. The GC method of repeating the analysis using a second chromatographic column of different polarity is recognised to provide only limited confirmatory evidence and a more definitive technique is preferred (Commission Recommendation 1999/333/EC, Article 63). Therefore, GC may function as a 'filter' when conducting simultaneous analyses of several pesticides (in this study, 125 pesticides). However, recent papers have reported detection of pesticides including metabolites of DDT in soil and plant tissues (Waliszewski *et al.*, 2004) and in cows milk (Armendariz *et al.*, 2004) using GC without further confirmation by an alternative identification method.

In the present study, the herbs tested were found not to contain pesticide residues above the detection limit of the method. Possible explanations include the breakdown of pesticides before harvesting or the possibility that the herbs were collected from isolated areas away from direct pesticide use. Although such herbs may contain less contaminant, the use of farmed herbs is currently being encouraged to support the sustainable use of medicinal plants (WHO, 2002). Also, it has been reported that when plants have been collected from the wild, "extracts suffer from a lack of reproducible bioactivity and chemical composition caused by the highly inducible, variable, and transitory nature of plant secondary metabolism" (Poulev *et al.*, 2003).

3.5. Concluding remarks

The authentication studies verified that 17 of the 24 TCM herbs were prepared from the correct plant species, as described in TCM. The selected herbs tested were found to be of adequate quality with respect to metal and pesticide residue content. However, the rhino horn sample analysed was contaminated with the metals Cd, Pb, Hg and Zn at concentrations above the set legal limits. In order to determine the suitability of the selected herbs as alternatives for rhino horn as well as bear bile, the herbs were tested for activity (Chapters 5, 6 and 7). The extraction and fractionation methods used to prepare the herbs for these purposes are described in Chapter 4.

CHAPTER 4. EXTRACTION, FRACTIONATION AND CHEMICAL ANALYSIS OF TRADITIONAL CHINESE MEDICINES

4.1. Introduction

The methods used to prepare herbal extracts and fractions, and to isolate compounds are presented in this Chapter. These methods apply to Chapters 5, 6 and 7. Chemical profiles of the samples were also obtained to ascertain the types of compounds in the extracts. Relevant biological activities associated with some of these compounds are also discussed in this chapter.

4.2. Experimental Procedure

4.2.1. Samples

Twenty-four herbs (described in Tables 3.2 to 3.4) and seven TCM prescriptions (compositions given in Table 2.7) were analysed. The chemical standards were rosmarinic acid, baicalin, baicalein, scutellarein, norwogonin and chrysin (purchased from Apin Chemicals Ltd., UK).

4.2.2. Extraction

In order to replicate the traditional methods of herbal preparations, hot water extractions were prepared. Powdered plant material (1g) of individual herbs was soaked in water for 1 hour before boiling for 45 minutes. The hot extracts were filtered through glass wool (traditionally a cloth filter would have been used). The extracts were then lyophilized (freeze-dried) and stored below 5^{o}C. Aqueous methanol (80%) and ethyl acetate extracts were subsequently prepared at room temperature following the method detailed in Section 3.2.2.2.

4.2.3. Bioactivity guided fractionations of Scutellaria baicalensis

4.2.3.1. Solvent extraction and column chromatography

Dried powdered root of *Scutellaria baicalensis* (35 g) was extracted in methanol (MeOH; 800 ml) by Soxhlet (65^{O}C) for 19 hours. The extract was concentrated to about 300 ml and partitioned in hexane (100 ml). Column chromatography using normal phase silica gel (10 g; 60A S-230/70 mesh, SL06SA4, YMC Co. Ltd) was performed on the dried methanol fraction (12 g). Prior to fractionation, analytical TLC was conducted on the extract to determine the most appropriate mobile phase (MP) for use in silica gel column chromatography. Different mixtures of MeOH, chloroform ($CHCl_3$), ethyl acetate (EtOAc), acetone (Me_2CO) and water (H_2O) were investigated. TLC plates (silica pre-coated aluminium-backed silica gel, $60F_{254}$ sheets, Merck, UK) were spotted with the crude extract and developed with either $CHCl_3$-EtOAc (1:1), $CHCl_3$-Me_2CO (9:1), $CHCl_3$-MeOH-H_2O (17:3:1), or $CHCl_3$-MeOH (9.5:0.5) to represent a range of solvent polarities. Based on the TLC results, a gradient elution procedure consisting of 10 combinations of $CHCl_3$, Me_2CO, MeOH and H_2O was devised (Table 4.1) for the silica gel column chromatography with increasing polarity from 100% $CHCl_3$ to 95% MeOH in H_2O. The total volume for each mobile phase system was 500 ml and 250 ml eluents were collected.

Table 4.1. Composition of mobile phase systems used for silica gel column fractionation of *Scutellaria baicalensis*.

Mobile phase system*	$CHCl_3$ (%)	Me_2CO (%)	MeOH (%)	H_2O (%)
1	100	-	-	-
2	98	2	-	-
3	95	5	-	-
4	90	10	-	-
5	80	20	-	-
6	50	50	-	-
7	50	25	25	-
8	50	20	25	5
9	50	-	45	5
10	-	-	95	5

*The total volume for each mobile phase system was 500 ml.

Equations 4.1 – 4.3 (William and Wilson, 1975) were used to calculate the volume of mobile phase required for efficient separation and the number of fractions to be collected. The height of the packed silica column was 23 cm with a radius of 2.5 cm. Therefore, the volume of mobile phase required was 519.6 ml (eqs. 4.1 and 4.2). The volume of MP used per elution in this study was 500 ml.

Column or bed volume = $V_c = A_cL = \pi r_c^2 L$ eq. 4.1

Where A_c is the internal cross-sectional area, L is the length of the packed part of the column and r_c is the inside radius of the column.

$V_c = 3.14 \times (2.5^2) \times 23 = 433$ ml

$V_o = 1.2 \times V_c$ eq. 4.2

Where V_o is the mobile phase volume.

$V_o = 1.2 \times 433 = 519.6$ ml

Equation 4.3 was used to calculate the number of fractions to be collected.

Number of fractions to be collected = $n = (5\%) \times V_c$ eq. 4.3

$n = (5/100) \times 433 = 21.7$

However, the actual number of fractions collected was 20. (The fractions were named SB followed by a number indicating the order of elution). For SB1 – SB10, acetone was used to transfer the residues formed in the collecting flasks (after solvent evaporation) to storage vials. The remainder of the fractions were soluble in MeOH. The extracts contained in the vials were brought to dryness on a heated concentrator (40 - 60°C) and stored below 5°C. Fraction SB4 was selected for further fractionation based on the results of NF-κB (see Fig. 6.3).

4.2.3.2. Fractionations of SB4 using TLC

From previous TLC analysis (section 4.1.3.1), compounds from *Scutellaria baicalensis* were found to separate well on TLC plates with $CHCl_3$-EtOAc (1:1) as MP. Therefore, preparative TLC was adopted as a method to fractionate SB4. Analytical TLC of SB4 using $CHCl_3$-EtOAc (1:1) gave a good separation of the compounds, but most of the compounds eluted quickly. In order to maintain good selectivity but improve resolution of the component bands, hexane was used to 'dilute' the MP and reduce mobility. Developed TLC plates using hexane-$CHCl_3$-EtOAc (1:2:2) for SB4 showed that the bands had moved nearer to the top of the plate resulting in better resolution compared to $CHCl_3$-EtOAc (1:1). Since the addition of hexane to the MP resulted in better resolution of the bands, its ratio was increased in the MP composition for preparative TLC where a mixture of hexane-$CHCl_3$-EtOAc (1:1:1) was used.

Preparative TLC was performed using four pre-coated glass-backed TLC plates (20 x 20 cm, SIL G-100, 1.0 mm silica gel, with fluorescent indicator UV254, Machery-Nagel, Germany). SB4 (0.37g) was re-dissolved in acetone (3.5 ml) containing a drop of $CHCl_3$ to give a homogenous solution. Using a Pasteur pipette, equal volumes of the extract were applied to four separate TLC plates. The plates were developed in two tanks (2 plates per tank) each containing 90 ml of hexane-$CHCl_3$-EtOAc (1:1:1). The plates were dried and the profiles of the bands observed under UV (254 nm) marking off nine identical and distinct sections on all four of the plates. Care was taken not to leave the plates under UV for a long period of time to avoid deterioration of the compounds. The nine sections from each of the four developed TLC plates were removed and the identical sections combined into nine separate 250 ml conical flasks and extracted with acetone (60 – 80 ml) for a minimum of 10 minutes. Each extract was filtered through a Whatman (# 1) filter and the residue washed with acetone (total of 20 ml). Each of the nine extracts was separately concentrated and transferred to pre-weighed vials (using acetone) and left to dry. The dried samples were weighed and the yields calculated.

4.2.3.3. Isolation of compounds from SB4v using HPLC

The fifth fraction (SB4v) obtained from the fractionation of SB4 was selected for further fractionation based on the results from the NF-κB tests (Section 6.3). Analytical HPLC analysis (Section 3.2.2.3) of SB4v showed the presence of three main peaks eluting at 21.26 minutes (peak 1), 21.87 minutes (peak 2) and 22.40 minutes (peak 3), with two other small peaks at 2.23 minutes and 22.41 minutes. Peak 2 was identified as chrysin by comparison with HPLC (UV-DAD) analysis of the pure compound. Semipreparative HPLC was conducted to collect the eluants corresponding to peaks 1, 2 and 3, separately, using a Merck LiChrosphor 100RP-18 column with 10 mm i.d. and a flow rate of 4.5 ml/min. Other chromatographic conditions described in Section 3.2.2.3 for the analytical HPLC (Section 3.2.2.3) were also applied for the isolation experiments.

4.2.3.4. Identification of compounds using NMR

Nuclear magnetic resonance (NMR) spectroscopy was employed to identify the compounds relating to peaks 1 and 3 and for further confirmation of peak 2. The NMR experiments were carried out using a Bruker Avance 400MHz NMR spectrometer at 30°C using $CDCl_3$ as solvent. NMR data were acquired using 1D ^{1}H (one-dimensional proton spectrum), $^{1}H,^{13}C$ HSQC (two-dimensional heteronuclear single quantum coherence spectroscopy) and HMBC (two-dimensional heteronuclear multiple bond correlation spectroscopy). Standard pulse sequences and parameters were used. The ^{13}C NMR spectral assignments were obtained by indirect detection as there was insufficient sample for the acquisition of directly observed 1D ^{13}C spectra. NMR data were acquired and interpreted by Dr N. Veitch (RBG, Kew).

4.2.4. Extraction and fractionation of Salvia miltiorrhiza and Lonicera japonica

Powdered *Salvia miltiorrhiza* (72 g) was extracted with MeOH (800 ml) using Soxhlet extraction (65^{o}C) for 17 hours and the extract was concentrated to dryness. The fractionation method outlined in Section 4.2.3.1 was followed using 11 g of crude extract. However, a different set of developing solvents (Table 4.2) were used based on results obtained from analytical TLC analysis of *S. miltiorrhiza*. A gradient elution of 8 combinations of $CHCl_3$, MeOH and H_2O with increasing polarity was used to collect 17 fractions.

Lonicera japonica (79 g) was extracted in 800 ml aqueous MeOH (80%) overnight at room temperature. The extract was filtered and concentrated. The fractionation method outlined in Section 4.2.3.1 was followed using 10 g of crude extract and the gradient mobile phase outlined in Table 4.2. A total of 18 fractions were collected.

Table 4.2 Mobile phase compositions used to fractionate *Salvia miltiorrhiza* and *Lonicera japonica*.

$CHCl_3$ (%)	MeOH (%)	H_2O (%)
100	-	-
87	12	1
73	24	3
64	36	5
64	36	5
50	40	10
20	65	15
-	85	15

The total volume for each mobile phase system was 500 ml.

4.2.5. Extraction of TCM prescriptions

Accurate proportions of herbs were weighed to form prescriptions in the ratio of 1:1 of the herbal compositions described in Table 2.7. The powdered mixtures of were extracted with aqueous MeOH (80%) or ethyl acetate at room temperature overnight as described in Section 3.2.2.2.

Hot water extractions of each of the five TCM prescriptions were prepared with and without rhino horn. Therefore, in total five herbal extracts with rhino horn and five herbal extracts without rhino horn as well as rhino horn extract were prepared. Mixtures of powdered herbs making up each herbal prescription (Table 2.7) were soaked for 30 minutes in water (in the ratio of 3:1 water-herb). Each herbal mixture was then brought to the boil using heating mantles, for 45 minutes. The hot extract was filtered through glass wool. Powdered rhino horn (6 g) was separately extracted with filtered water (100 ml) by boiling for 30 minutes. After filtering, $1/6^{th}$ of the total volume of five herbal extracts was individually mixed with $1/6^{th}$ of rhino horn extract, leaving $1/6^{th}$ of rhino horn extract for testing. All the extracts were freeze-dried.

4.2.5.1. Fractionation of TCM prescriptions

Lyophilized crude hot water extracts of the prescriptions qing ying tang (2.36 g) and zhi zhi jin hua wan (3g) were separately re-constituted in 150 ml aqueous MeOH (5%). In separate experiments, the extracts were fractionated using flash chromatography. A stepwise gradient elution system was used starting with 5% MeOH and ending with 100% MeOH in volumes of 600 ml (Table 4.3). After packing the column (with reversed phase silica gel) and before loading the sample the initial mobile phase of 600 ml of aqueous MeOH (5%) was used to condition the column. This was followed by another 600 ml of 5% MeOH and sequentially by the other mobile phase compositions listed in Table 4.3. Fractions were collected in volumes of 300 ml and concentrated. Twenty-three and 24 fractions were collected for zhi zhi jin hua wan and qing ying tang, respectively.

4.2.6. HPLC (UV-DAD) analysis

The herbal extracts and fractions, in addition to reference samples of rosmarinic acid, baicalin, baicalein, scutellarein, norwogonin and chrysin were analysed using HPLC (UV-DAD) as detailed in Section 3.2.2.3.

Table 4.3. Composition of mobile phase systems used for the fracti onations of TCM prescriptions using flash chromatography.

Mobile phase system	MeOH (%)	H_2O (%)
1	5	95
2	10	90
3	15	85
4	20	80
5	30	70
6	40	60
7	50	50
8	60	40
9	70	30
10	80	20
11	100	-

* Each mobile phase system was 600 ml.

4.3. Results

Extracts of 24 herbs (Table 4.4), fractions and compounds from *Scutellaria baicalensis* (Tables 4.5 and 4.6), fractions from *Salvia miltiorrhiza* (Table 4.7) and *Lonicera japonica* (Table 4.8) were prepared for activity tests (Chapters 5, 6 and 7). In addition, extracts from 12 TCM prescriptions, as well as rhino horn extracts (Table 4.9), fractions from the TCM prescriptions, qing ying tang (Table 4.10) and zhi jin hua (23 fractions) were also prepared.

Three flavonoid compounds were isolated and identified from dried roots of *S. baicalensis*. The NMR data for wogonin and orxylin A are give in Appendix VIII. The UV spectra of these compounds as well as three other constituents (reference samples) of *S. baicalensis* are given in Fig. 4.1. Chrysin, wogonin, oroxylin A, baicalein, baicalin and norwogonin were identified from the characteristic UV profiles as being present in some fractions of *S. baicalensis* Fig. 4.1. Also, comparison of the UV spectra of rosmarinic acid (λmax = 237.9, 290sh, 300sh, 330 nm) with the profiles of fractions obtained from a methanolic extract of *S. miltiorrhiza* indicated the presence of this rosmarinic acid in fractions 6, 7 and 8.

Table 4.4. Extracts of 24 TCM herbs investigated in biological activity tests.

TCM samples (the names of plant species confirmed in this study are given in italics)	Anti-bacterial tests, 100 µg Results are shown in Table 5.2	NF-κB inhibition, 100 µg/ml Results are shown in Figures 6.2, 6.3 & 6.7	Cytochrome P450s, 100 µg/ml Results are shown in Figures 7.3 & 7.6
1. *Scrophularia ningpoensis* Hemsl. (xuan shen)	ethyl acetate	water	water
2. *Rehmannia glutinosa* Steud. (sheng di huang)	ethyl acetate	water	water
3. *Paeonia suffruticosa* Andr. (mu dan pi)	nt	water	water
4. *Paeonia lactiflora* Pall. (chi shao yao)	ethyl acetate	water	water
5. Arnebiae seu Lithospermi Radix (zi cao)	ethyl acetate	nt	nt
6. Isatidis Radix (ban lan gen)	ethyl acetate	water	water
7. *Lonicera japonica* Thunb. (jin yin hua)	ethyl acetate / fractions	water	water
8. *Forsythia suspensa* Vahl (lian qiao)	ethyl acetate	nt	nt
9. *Salvia miltiorrhiza* Bge. (dan shen)	ethyl acetate / aq. MeOH (80%)	fractions	fractions
10. *Anemarrhena asphodeloides* Bge. (zhi mu)	ethyl acetate	water	water
11. *Gardenia jasminoides* Ellis (zhi zi)	ethyl acetate	nt	water
12. *Scutellaria baicalensis* Georgi (huang qin)	ethyl acetate	water / ethyl acetate / MeOH	water
13. Coptidis Rhizoma (huang lian)	ethyl acetate	water	water
14. *Phellodendron amurense* Rupr. (huang bai)	ethyl acetate	water	water
15. *Andrographis paniculata* Nees (chuan xin lian)	ethyl acetate	water	water
16. Rhei Radix et Rhizoma (da huang)	ethyl acetate / aq. MeOH (80%)	water	nt
17. Ophiopogonis Radix (mai *men dong*)	ethyl acetate	nt	nt
18. *Lophatherum gracile* Brongn. (dan zhu ye)	ethyl acetate	water	nt
19. *Platycodon grandiflorum* A.DC. (jie geng)	ethyl acetate	water	water
20. *Glycyrrhiza uralensis* Fisch. (gan cao)	ethyl acetate	water	water
21. Sojae Praeparatum Semen (dan dou chi)	ethyl acetate	water	nt
22. Trichosathes Radix (tian hua fen)	ethyl acetate	water	nt
23. Acori Graminei Rhizoma (chang pu)	ethyl acetate	water	water
24. Nelumbinis Semen (lian zi xin)	ethyl acetate	nt	nt

The TCM samples were extracted with ethyl acetate, methanol (MeOH), aqueous MeOH or water as described in Section 4.2.2. nt = not tested.

Table 4.5. Fractions obtained from *Scutellaria baicalensis* (SB), the yields obtained and the assays used to investigate their biological activity.

Fractions obtained from a methanolic extract of SB (SBM1)	Dry mass (mg)	Yield % from SBM	Yield % from dried herb	Anti-bacterial tests 10 µg	NF-κB inhibition 100 µg/ml	Cytochrome P450s 100 µg/ml
SB 1	6.3	0.05	0.02	nt	See Fig. 6.4	nt
SB 2	8.9	0.07	0.03	nt	See Fig. 6.4	nt
SB 3	35.5	0.30	0.10	nt	See Fig. 6.4	nt
SB 4	381.3	3.18	1.08	nt	See Fig. 6.4	nt
SB 5	87.1	0.73	0.25	See Table 5.3	See Fig. 6.4	nt
SB 6	23.4	0.20	0.07	See Table 5.3	See Fig. 6.4	nt
SB 7	17.9	0.15	0.05	See Table 5.3	See Fig. 6.4	nt
SB 8	20.9	0.17	0.06	See Table 5.3	See Fig. 6.4	nt
SB 9	22.8	0.19	0.06	See Table 5.3	See Fig. 6.4	nt
SB 10	21.1	0.18	0.06	See Table 5.3	See Fig. 6.4	nt
SB 11	17.4	0.15	0.05	See Table 5.3	See Fig. 6.4	nt
SB 12	153.3	1.28	0.43	See Table 5.3	See Fig. 6.4	nt
SB 13	192.2	1.60	0.54	See Table 5.3	See Fig. 6.4	nt
SB 14	591.2	4.93	1.67	See Table 5.3	See Fig. 6.4	See Fig. 7.4
SB 15	1470	12.25	4.16	See Table 5.3	See Fig. 6.4	nt
SB 16	526.3	4.39	1.49	See Table 5.3	See Fig. 6.4	nt
SB 17	585.2	4.88	1.66	See Table 5.3	See Fig. 6.4	nt
SB 18	1110	9.25	3.14	See Table 5.3	See Fig. 6.4	nt
SB 19	590	4.92	1.67	See Table 5.3	See Fig. 6.4	nt
SB 20	5950	49.58	16.85	See Table 5.3	See Fig. 6.4	nt
Total	11810.8 mg (11.81 g)	98.4	33.4			

[1]Dried herbs of *Scutellaria baicalensis* (35.3 g) was extracted to obtain 12.11 g of SBM. SBM (12 g) was then fractionated as detailed in Section 4.2.3.1 the yield of SBM from the dried herbs was 34.3%. nt = not tested.

Table 4.6. Fractions obtained from SB4 and compounds from HPLC of SB4v, their yields and assays used to investigate their biological activity.

Fractions from *S. baicalensis* fraction 4 (SB4)	Dry mass (mg) (from 0.37 g of SB 4)	Yield (%)	Biological activity tests	
			NF-κB inhibition 100 μg/ml	cytochrome P450s 20 μg/ml
SB4i	14.8	4.0	nt	nt
SB4ii	7.8	2.1	nt	nt
SB4iii	16.3	4.4	nt	nt
SB4iv	25	6.8	nt	nt
SB4v	62	16.8	See Fig. 6.4	See Fig. 7.4
SB4vi	38.3	10.4	See Fig. 6.4	nt
SB4vii	9.5	2.6	nt	nt
SB4viii	63.1	17.1	See Fig. 6.4	nt
SB4ix	13.6	3.7	nt	nt
Total	250.4	67.7		
Compounds from SB4v (18.7 mg)			(up to 100 μg/ml)	
Chrysin	2.4	12.8	See Fig. 6.5	See Fig. 7.4
Oroxylin A	4.1	21.9	See Fig. 6.5	nt
Wogonin	2.0	10.7	See Fig. 6.5	nt
Total	8.5	45.5		

Other compounds from S. baicalensis tested in both the NF-κB and cytochrome P450 assays were baicalin, baicalein and scutellarein.
nt = not tested in the assay.

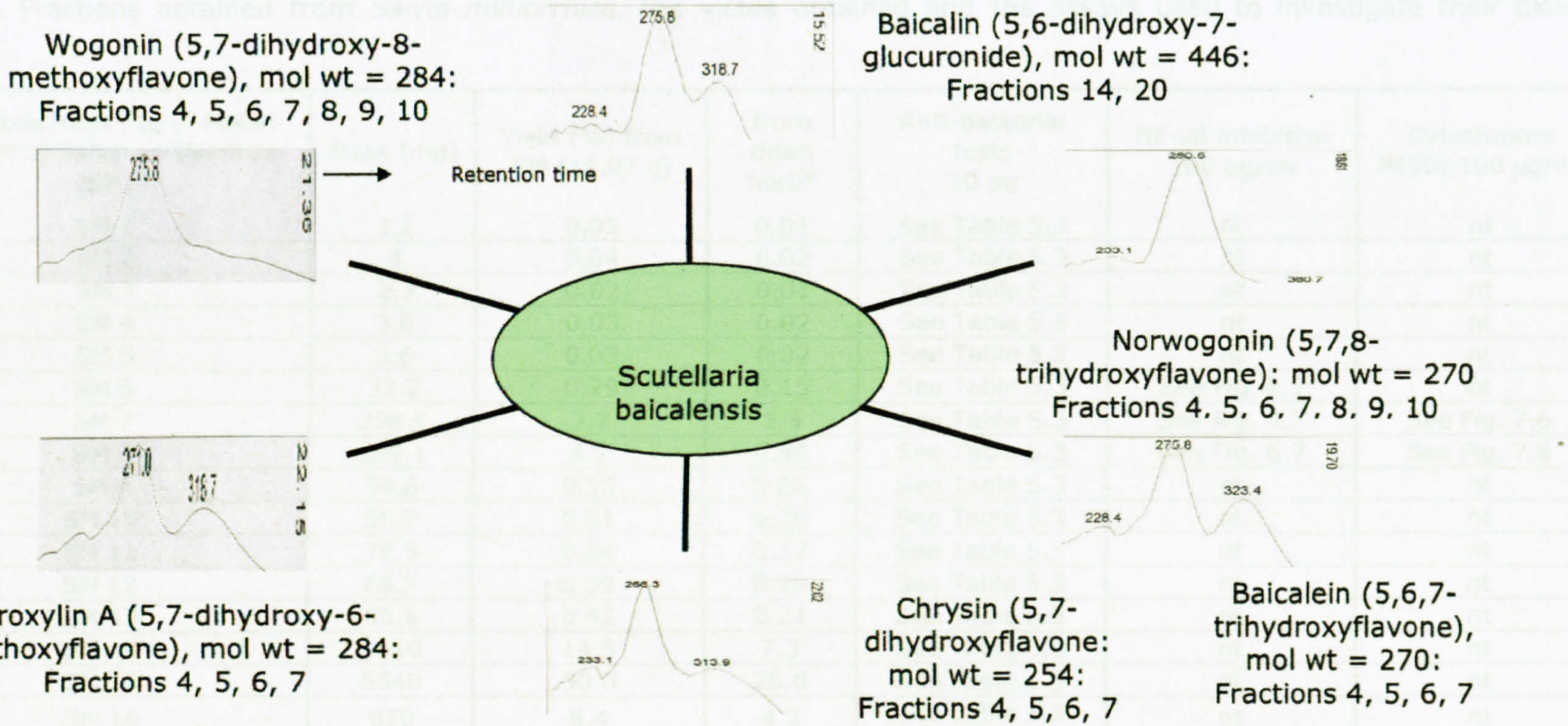


Fig. 4.1. Ultraviolet (UV) spectra of some compounds identified in biologically active fractions of *Scutellaria baicalensis*.
HPLC (UV-DAD) profiles of baicalin, norwogonin, baicalein and chrysin (obtained from reference standards) and wogonin and oroxylin A (isolated from *S. baicalensis*) were compared to those obtained for fractions of methanolic extract of *S. baicalensis*. With the exception of fractions 14 and 20, all the fractions listed in the diagram, as well as chrysin, wogonin, baicalein and oroxylin A demonstrated potential anti-inflammatory effects by showing inhibitory NF-κB activity (Figs. 6.4, 6.5 and Section 6.4).

Table 4.7. Fractions obtained from *Salvia miltiorrhiza*, the yields obtained and the assays used to investigate their biological activity.

Fractions from 11g of MeOH extract of *Salvia miltiorrhiza* (SM)	Mass (mg)	Yield (%) from SM (11.07 g)	from dried herb*	Anti-bacterial tests 10 µg	NF-κB inhibition 100 µg/ml	Cytochrome P450s 100 µg/ml
SM 1	3.2	0.03	0.01	See Table 5.3	nt	nt
SM 2	4	0.04	0.02	See Table 5.3	nt	nt
SM 3	2.3	0.02	0.01	See Table 5.3	nt	nt
SM 4	3.6	0.03	0.02	See Table 5.3	nt	nt
SM 5	3.6	0.03	0.02	See Table 5.3	nt	nt
SM 6	32.2	0.29	0.15	See Table 5.3	See Fig. 6.7	nt
SM 7	298.9	2.7	1.3	See Table 5.3	See Fig. 6.7	See Fig. 7.6
SM 8	189.1	1.7	0.85	See Table 5.3	See Fig. 6.7	See Fig. 7.6
SM 9	54.8	0.50	0.25	See Table 5.3	nt	nt
SM 10	56.7	0.51	0.26	See Table 5.3	nt	nt
SM 11	70.5	0.64	0.32	See Table 5.3	nt	nt
SM 12	65.3	0.59	0.29	See Table 5.3	nt	nt
SM 13	46.6	0.42	0.21	See Table 5.3	nt	nt
SM 14	1610	14.5	7.3	See Table 5.3	nt	nt
SM 15	5540	50.0	25.0	See Table 5.3	nt	nt
SM 16	930	8.4	4.2	See Table 5.3	nt	nt
SM 17	25.4	0.23	0.11	See Table 5.3	nt	nt
Total	8936.2	80.7	40.3			

*A yield of 49.9% was achieved for the extraction of SM from the dried herbs of *Salvia miltiorrhiza*. The fractionation method is described in Section 4.2.4. nt = not tested.

Table 4.8. Fractions obtained from *Lonicera japonica*, the yields obtained and the assay used to investigate their biological activity.

Fractions from 10 g extract of *Lonicera japonica*	Mass (mg)	Yield (%) from extract	Yield (%) from dried herb*	Anti-bacterial tests 10 µg
LJ 1-5	1.2	0.012	0.003	See Table 5.3
LJ 6	1.7	0.017	0.005	See Table 5.3
LJ 7	157	1.57	0.443	See Table 5.3
LJ 8	323	3.23	0.911	See Table 5.3
LJ 9	1260	12.6	3.552	See Table 5.3
LJ 10	1070	10.7	3.017	See Table 5.3
LJ 11	510	5.1	1.438	See Table 5.3
LJ 12	364	3.64	1.026	See Table 5.3
LJ 13	404	4.04	1.139	See Table 5.3
LJ 14	384	3.84	1.083	See Table 5.3
LJ 15	2990	29.9	8.43	See Table 5.3
LJ 16	1360	13.6	3.834	See Table 5.3
LJ 17	286	2.86	0.806	See Table 5.3
LJ 18	37	0.37	0.104	See Table 5.3
Total	9147.9	91.479	25.8	

*A yield of 28.2% was achieved for the extraction of LJ from the dried herbs *Lonicera japonica*. The fractionation method is described in Section 4.2.4.

Table 4.9. Extracts of rhino horn and TCM prescriptions investigated in biological activity tests.

TCM prescriptions	Anti-bacterial tests 100 µg; Results are shown in Table 5.4	NF-κB inhibition, 100 µg/ml; Results are shown in Fig. 6.6	CYP450, 100 µg/ml; Results are shown in Fig. 7.5
Rhino horn	water / ethyl acetate	water	water
Zhi zi jin hua	water / ethyl acetate	water	nt
Qingwen baidu yin plus rhino horn	water / ethyl acetate	water	nt
Qingwen baidu yin without rhino horn	water / ethyl acetate	water	nt
Xi jiao dihuang tang plus rhino horn	water / ethyl acetate	water	nt
Xi jiao dihuang tang without rhino horn	water / ethyl acetate	water	nt
Qing ying tang plus rhino horn	water / ethyl acetate	water	water
Qing ying tang without rhino horn	water / ethyl acetate	water	water
Sheng xi dan plus rhino horn	water / ethyl acetate	water	water
Sheng xi dan without rhino	water / ethyl acetate	water	water
Qing gong tang plus rhino horn	water / ethyl acetate	water	nt
Qing gong tang without rhino horn	water / ethyl acetate	water	nt
Prescription X	water / ethyl acetate	water	water

The extraction methods are described in Sections 3.2.2.2 and 4.2.5. nt = not tested.

Table 4.10. Fractions obtained from qing ying tang (without rhino horn; QYT) investigated in biological activity tests.

Fractions from water extract of qing ying tang (QYT)	Antibacterial tests, 20 µg/ml	NF-κB inhibition, 100 µg/ml
QYT 1	nt	nt
QYT 2	See Table 5.4	nt
QYT 3	See Table 5.4	nt
QYT 4	See Table 5.4	nt
QYT 5	See Table 5.4	nt
QYT 6	See Table 5.4	nt
QYT 7	See Table 5.4	nt
QYT 8	See Table 5.4	nt
QYT 9	See Table 5.4	See Fig. 6.7
QYT 10	See Table 5.4	nt
QYT 11	See Table 5.4	nt
QYT 12	See Table 5.4	nt
QYT 13	See Table 5.4	nt
QYT 14	See Table 5.4	nt
QYT 15	See Table 5.4	See Fig. 6.7
QYT 16	See Table 5.4	See Fig. 6.7
QYT 17	See Table 5.4	See Fig. 6.7
QYT 18	See Table 5.4	nt
QYT 19	See Table 5.4	nt
QYT 20	See Table 5.4	nt
QYT 21	See Table 5.4	nt
QYT 22	See Table 5.4	nt
QYT 23	See Table 5.4	nt
QYT 24	See Table 5.4	nt
QYT 25	See Table 5.4	nt

nt = not tested.

4.4. Discussion

Chemical profiles were obtained for the extracts and fractions prepared for activity tests. This procedure was used to identify compounds with potential biologically active properties, by comparing the UV spectra of extracts with those of reference standards and/or with reference UV spectra from the literature (Appendix V). The compounds identified in 80% aqueous methanol extracts of some of the herbs are given in Appendix VII.

Bioactivity guided fractionations of *Scutellaria baicalensis* (Labiatae), led to the isolation and identification of three flavonoid compounds (oroxylin A, wogonin and chrysin). Recently, Li et al. (2004) published a review of different analytical methods used to separate active compounds from *S. baicalensis*. In the current study, the comparison of HPLC (UV-DAD) profiles of the initial 20 fractions obtained from *S. baicalensis* (Table 4.5) with those of the isolated compounds and other reference compounds indicated the presence of oroxylin A, wogonin and chrysin, as well as baicalein (reference sample) in fractions demonstrating NF-κB inhibitory activities (Figs. 4.1 and 6.4). The anti-inflammatory potential of oroxylin A, wogonin, chrysin and baicalein are documented (Chen *et al.*, 2000; Nakamura *et al.*, 2003; Chen *et al.*, 2004). Results from this study have shown that wogonin, chrysin and baicalein exert anti-inflammatory effects through the inhibition of NF-κB activity (Fig. 6.5 and Section 6.4).

Another compound with known relevant pharmacological effects that was detected in some herbal fractions in this study was rosmarinic acid, a caffeic acid derivative. The UV profile (λmax = 237.9, 290sh, 300sh, 330 nm) of a reference sample of rosmarinic acid indicated that the compound was present in fractions 6, 7 and 8 of the *Salvia miltiorrhiza* (Lamiaceae) extract, along with several other compounds. These fractions of *S. miltiorrhiza* had demonstrated anti-bacterial activity (Table 5.3) and potent inhibitory NF-κB activity (Fig. 6.7). Rosmarinic acid was not tested in the NF-κB assays conducted in this study. However, rosmarinic acid has been reported to possess anti-inflammatory (Osakabe *et al.*, 2004), anti-oxidant (Bors *et al.*, 2004) and chemoprotective (Chlopcikova *et al.*, 2004) properties. Rosmarinic acid has been isolated from *Rosmarinus officinalis* (Petersen and Simmonds, 2003) from the Lamiaceae family. It is also found in some other species of *Salvia* including *S. officinalis* and many other species of the Lamiaceae and Boraginaceae plant families (Patersen and Simmonds, 2003). Although plant materials used to prepare medicinal herbs often contain a wide range of compounds, the identification of an active compound may be useful in targeting species for future studies.

4.5. Concluding Remarks

The 24 herbs, 12 TCM prescriptions and rhino extracts were tested in either anti-bacterial (Chapter 5), NF-κB (Chapter 6) or/and cytochrome P450 (Chapter 7) tests. Fractions obtained from the three herbs (*Scutellaria baicalensis*, *Salvia miltiorrhiza* and *Lonicera japonica*) and two TCM prescriptions were also tested, in addition to some compounds isolated from *Scutellaria baicalensis*.

Chapter 5. Anti-bacterial Studies

5.1. Introduction

Fever (or pyrexia) in the concept of Western medicine is the elevation of body temperature above the normal (36.5^{o}C) and is often a clinical sign of infection and inflammation (Dinarello *et al.*, 1999). In humans the most common fever is pathogenic fever caused by bacteria.

Rhino horn and bear bile are animal products used in TCM to relieve inflammatory conditions and fever (Hsu *et al.*, 1986; Bensky and Gamble, 1993). In TCM, herbs used in the treatment of fever and inflammation are often also attributed with anti-bacterial properties (Hsu *et al.*, 1986; Bensky and Gamble, 1993). Therefore, in this study, herbs selected for investigation as herbal alternatives to bear bile and rhino horn were also tested with respect to their inhibitory effect on Gram-negative and Gram-positive bacteria. The assays were conducted at the Jodrell Laboratory, Royal Botanic Gardens, Kew.

5.2. Experimental Procedure

5.2.1. Samples

The descriptions of herbs and TCM prescriptions as well as rhino horn extracts tested in the anti-bacterial assay are given in Table 5.1. The extraction and fractionation procedures used to prepare the samples are described in Chapter 4.

Table 5.1. A list of herbs, herbal fractions, TCM prescriptions and rhino horn extracts tested in the anti-bacterial assay

Plant species and Pin yin name (Latin names (in italics) are given for herbs for which the plant species have been verified (see Section 3.2)
Phellodendron amurense Rupr. (Huang bai)
Coptis Rhizoma (huang lian)
Rhei Radix et Rhizoma (Da huang)
Anemarrhena asphodeloides Bge. (zhi mu)
Andrographis paniculata Nees (chuan xin lian)
Trichosanthis Radix (Tian hua fen)
Lophatherum gracile Brongn. (Dan zhu ye)
Acori Graminei Rhizoma (Chang pu)
Paeonia lactiflora Pall. (chi shao yao)
Isatidis Radix (ban lan gen)
Sojae Praeparatum semen (Dan dou chi)
Rehmannia glutinosa Steud. (sheng di huang)
Scrophularia ningpoensis Hemsl. (xuan shen)
Platycodon grandiflorum A.DC. (jie geng)
Glycyrrhiza uralensis Fisch. (gan cao)
Ophiopogonis Radix (mai men dong)
Arnebiae seu Lithospermi Radix (z i cao)
Gardenia jasminoides Ellis (zhi zi)
Forsythia suspensa Vahl (lian qiao)
Nelumbinis Semen (lian zi xin)
Lonicera japonica Thunb. (jin yin hua)
Salvia miltiorrhiza Bge. (dan shen)
Scutellaria baicalensis Georgi (huang qin)
Fractions from methanolic extract of *Lonicera japonica* (fractions 1– 18)
Fractions from of methanolic extract of *Salvia miltiorrhiza* (fractions 1–17)
Fractions from methanolic extract of *Scutellaria baicalensis* (fractions 3, 5–20)
Prescription X
Rhino horn, xi jiao
Zhi zi jin hua
Xi jiao dihuang tang without rhino horn
Xi jiao dihuang tang with rhino horn
Sheng xi dan without rhino horn
Sheng xi dan plus rhino horn
Qing gong tang without rhino horn
Qing gong tang with rhino horn
Qingwen baidu yin without rhino horn
Qingwen baidu yin with rhino horn
Qing ying tang without rhino horn
Qing ying tang with rhino horn
Fractions from flash chromatography of qing ying tang (fractions 2–25)
Fractions from flash chromatography of zhi zi jin hua (fractions 1–23)

5.2.2. Sample preparation

The extraction procedures for the herbs, rhino horn and TCM prescriptions are described in Chapter 4. The dried MeOH/H_2O (80%) herb extracts were

re-constituted in MeOH and the dried ethyl acetate herb extracts were re-constituted in ethyl acetate to give solutions with concentrations of 5 mg/ml. Two separate solutions were prepared for each of the dried water prescription extracts, using either ethyl acetate or MeOH to give a final concentration of 5 mg/ml. Dried fractions obtained from column chromatography of *Scutellaria baicalensis* (Section 4.2.3), *Salvia miltiorrhiza* (Section 4.2.4) and *Lonicera japonica* (Section 4.2.4) were prepared in MeOH to give concentrations of 0.5 mg/ml each. Fractions obtained from water extracts of qing ying tang (23 fractions) and zhi zi jin hua (23 fractions) (as described in Section 4.2.5.1) were prepared at concentrations of 1 mg/ml in MeOH.

5.2.3. TLC analysis

Anti-bacterial tests were conducted using the TLC based direct agar overlay bioautography technique to determine qualitative inhibitory effects of plant extracts on the Gram-negative bacterium, *Pseudomonas syringae* and the Gram-positive bacterium, *Bacillus subtilis*. For the herb extracts, 20 µl of the 5 mg/ml solution was applied onto the TLC plates (20 x 10 cm^2, pre-coated aluminium-backed silica gel, $60F_{254}$ sheets, product number 1.05554, Merck, Germany), which can be interpreted as 100 µg of extract tested. Herbal fractions were tested by applying 20 µl of 1 mg/ml or 0.5 mg/ml solution onto the TLC plate resulting in 20 µg or 10 µg, respectively, of sample being tested. The TLC plates were developed in a tank containing one of the following solvent systems: chloroform-acetone (4:1) or (17:3) or chloroform-acetone-water (7:3:1) according to the suitability of the solvents for efficient separation. Developed plates were observed under UV light (254 nm and 355 nm). One of the developed TLC plates was subjected to chemical analysis and two were used to conduct anti-bacterial tests. The TLC plate for chemical analysis was sprayed evenly with *p*-anisaldehyde (0.5 ml in 50 ml HOAc and 1 ml conc. H_2SO_4) and the chemical profile of the sprayed plate was examined under UV light (only at 355nm since *p*-anisaldehyde absorbs UV at 254 nm). After heating, plates were re-examined under UV light (355 nm).

5.2.4. Anti-bacterial tests

The method for assessing anti-bacterial activity was a modification of that described by Rehalison *et al.* (1991). Chloramphenicol (C0378, Sigma) was used as the control drug. Two of the developed TLC plates were fixed onto separate culture dishes and chloramphenicol (3 µg/ml) was applied to a solvent-free area on each TLC plate. Distilled water (100 ml) was used to dissolve 2.8 g of nutrient agar (CM3, Oxoid, UK) and brought to boiling whilst stirring occasionally. A small colony of cultured bacteria of either *Pseudomonas syringae* (ID No. IMI347448, CABI Bioscience, UK) or *Bacillus subtilis* (ID No. IMI347329, CABI Bioscience, UK) was suspended in water and added to 50 ml nutrient agar solution (approximate temperature 50^{o}C) to form a seeded medium. This medium was used immediately after preparation, as an overlay on the TLC plate to form bioautograms with a layer of approximately 1 mm thickness. The bioautograms were sealed and incubated overnight (37^{o}C, 100% relative humidity). After incubation, the bioautograms were stained with *p*-iodonitrotetrazolium violet (2-[4-indophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride, 126H5042, Sigma, USA) diluted in ethanol (0.5 mg/ml) and then diluted ten-fold in H_2O. The dishes were incubated at 37^{o}C for a further two hours and then visually examined. Areas on the plate with inhibited or reduced bacterial growth appeared as white spots against a pink background. In the interpretation of the results, inhibition of bacterial growth by one or more separated TLC bands was taken as positive. A 'stronger' inhibition was said to have occurred if several bands caused inhibition and/or a comparatively larger area of inhibition occurred.

5.3. Results

Chloramphenicol, the positive control drug totally inhibited the growth of both *P. syringae* and *B. subtilis*. The areas on the TLC plates that were solvent and chemical free were used as negative controls and total growth

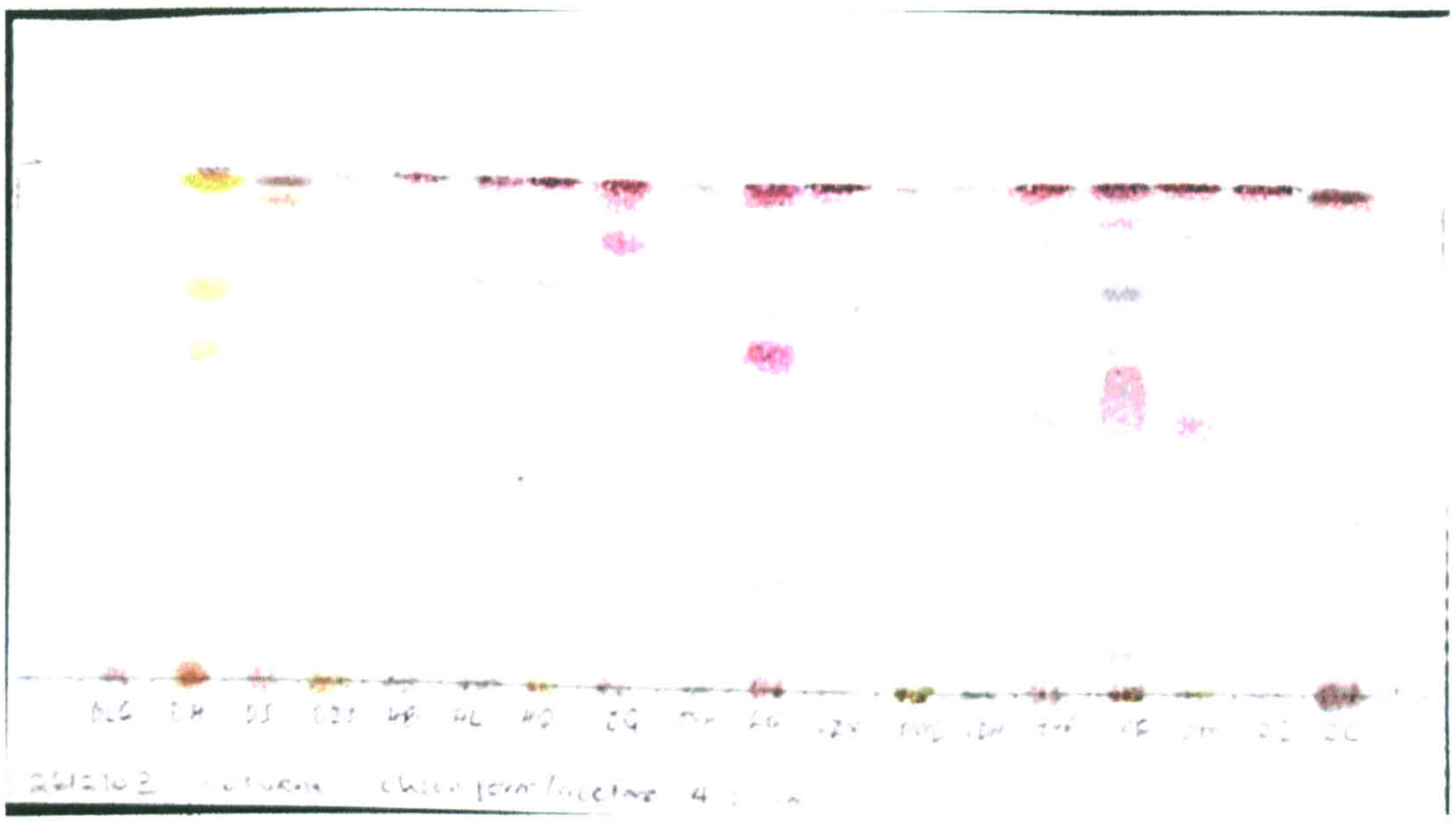
by both bacteria were observed in these areas. For each set of experiments, three replicates of developed TLC plates were viewed under UV (254 and 355 nm) to verify that they had similar chemical profiles as indicated by similar bands of separated compounds.

5.3.1. Anti-bacterial activity of TCM herbs

Aqueous MeOH (80%) extracts (100 μg) as well as ethyl acetate extracts (100 μg) of Rhei Radix et Rhizoma (da huang; DH) showed some inhibitory effect on the growth of both *B. subtilis* and *P. syringae*. Also, inhibitory spots were observed for the aqueous MeOH (80%) extract (100 μg) of *Salvia miltiorrhiza* (dan shen; DS) on both *B. subtilis* and *P. syringae* plates and the ethyl acetate extract (100 μg) also showed inhibitory effect on *B. subtilis*. Compared to several of the herbs tested, bands from ethyl acetate extract of Rhei Radix et Rhizoma and *Salvia miltiorrhiza* showed stronger inhibition on the growth of *B. subtilis* as illustrated in Fig. 5.1. With the exception of Nelumbinis Semen (lian zi xin) and Trichosanthis Radix (tian hua fen, THF) which had no apparent effect on the bacteria, the other 14 herbs showed some inhibition of *B. subtilis* (Fig. 5.1). In other tests ethyl acetate extracts of Acori Graminei Rhizoma (Chang pu), *Paeonia lactiflora* (chi shao yao), *Andrographis paniculata* (chuan xin lian) and *Glycyrrhiza uralensis* (gan cao) showed no inhibitory effect on *B. subtilis*, whereas Sojae Praeparatum semen (dan dou chi) demonstrated some inhibitory effect against *B. subtilis* (Table 5.2).

Fractions (10 μg) obtained from column chromatography of *Scutellaria baicalensis* and *Salvia miltiorrhiza* also showed inhibitory activity towards both *B. subtilis* and *P. syringae* (Table 5.2). Fractions LJ11, LJ12 and LJ13 (10 μg), from *Lonicera japonica* showed selective inhibition of *B. subtilis* (Table 5.3).

A



B

Fig. 5.1. TLC plates showing bands of separated compounds of 18 herbs (A) compared to growth inhibition of *Bacillus subtilis* on an identical TLC plate (B). Inhibited bacterial growth is shown as white areas in a pink background. The arrows indicate the columns representing Rhei Radix et Rhizoma (da huang; DH) and *Salvia miltiorrhiza* (dan shen; DS).

Table 5.2. Anti-bacterial effects of herbal extracts

Ethyl acetate extracts (100 μg of dried extract on TLC plate) and abbreviations used in Fig. 5.1.	Growth inhibition of *Bacillus subtilis*
Salvia miltiorrhiza Bge. (dan shen; DS)*	+
Rhei Radix et Rhizoma (da huang; DH)*	+
Phellodendron amurense Rupr. (huang bai; HB)	+
Coptis Rhizoma (huang lian; HL)	+
Anemarrhena asphodeloides Bge. (zhi mu; ZM)	+
Lophatherum gracile Brongn. (dan zhu ye; DZY)	+
Isatidis Radix (ban lan gen; BLG)	+
Rehmannia glutinosa Steud. (sheng di huang; SDH)	+
Scrophularia ningpoensis Hemsl. (xuan shen; XS)	+
Platycodon grandiflorum A.DC. (jie geng; JG)	+
Ophiopogonis Radix (mai men dong; MMD)	+
Arnebiae seu Lithospermi Radix (z i cao; ZC)	+
Gardenia jasminoides Ellis (zhi zi; ZZ)	+
Forsythia suspensa Vahl (lian qiao; LQ)	+
Lonicera japonica Thunb. (jin yin hua; JYH)	+
Scutellaria baicalensis Georgi (huang qin; HQ)	+
Sojae Praeparatum semen (dan dou chi)	+
Nelumbinis Semen (lian zi xin, LZX)	-
Trichosanthis Radixi (Tian hua fen; THF)	-
Andrographis paniculata Nees (chuan xin lian)	-
Acori Graminei Rhizoma (chang pu)	-
Paeonia lactiflora Pall. (chi shao yao)	-
Glycyrrhiza uralensis Fisch. (gan cao)	-

+ indicates that inhibitory spots were detected; – indicates no inhibition
*Aqueous MeOH (80%) extract of *Salvia miltiorrhiza* (100 μg) as well as Rhei Radix et Rhizoma (100 μg) inhibited the growth of both *Bacillus subtilis* and *Pseudomonas syringae*. Results for other tests on *P. syringae* are not presented due to uneven bacterial growth in replicate analyses.

Table 5.3. Anti-bacterial activity of fractionated herbal extracts as prepared by column chromatography

Fractions from methanolic extracts (10 μg)	Fractions tested	Fractions with inhibitory activity against *Pseudomonas syringae*	Fractions with inhibitory activity against *Bacillus subtilis*
Scutellaria baicalensis (SB)	SB3, SB5 – 20	SB5 – 12	SB12 (strong inhibition)
Salvia miltiorrhiza (SM)	SM1 – 17	SM3 - 7	SM3 - 7 SM8 (strong inhibition)
Lonicera japonica (LJ)	LJ1 – 5 (combined), LJ6 – 18	none detected	L11- 13

5.3.2. Anti-bacterial activity of rhino horn and TCM prescriptions

Dried hot water extracts of rhino horn and TCM prescriptions, re-dissolved in either ethyl acetate or methanol (100 μg), were tested for inhibitory effects on the growth of either *B. subtilis* or *P. syringae*. The results are summarised in Table 5.4. In addition, results for the anti-bacterial activity of fractions (20 μg) obtained from water extracts of qing ying tang (24 fractions) and zhi zi jin hua (23 fractions) are also presented in Table 5.4.

Table 5.4. Anti-bacterial activity of rhino horn and TCM prescriptions

Rhino horn and TCM prescriptions (100 μg)	Anti-bacterial tests	
	Pseudomonas syringae	*Bacillus subtilis*
Rhino horn	-	-
Qing ying tang with RH (QYTRH)	-	+ (W-E)
Qing ying tang without rhino horn (QYT)	-	+ (W-E, W-M)
Fractions from water extract of QYT (QYT2 – 25)	-	+ (F9, F15, F16, F17)
Sheng xi dan plus rhino horn (SXDRH)	+ (W-E)	+ (W-E, W-M)
Sheng xi dan without rhino horn (SXD)	+ (W-E)	+ (W-E, W-M)
Qing gong tang plus rhino horn (QGTRH)	-	+ (W-M)
Qing gong tang without rhino horn (QGT)	+ (W-E)	+ (W-M)
Qingwen baidu yin plus rhino horn (QWBYRH)	-	-
Qingwen baidu yin without rhino horn (QWBY)	-	-
Xi jiao dihuang tang plus rhino horn (XJDHTRH)	-	-
Xi jiao dihuang tang without rhino horn (XJDHT)	-	-
Zhi zi jin hua (ZZJH)	-	-
Fractions from water extract of ZZJH (ZZJH1 – 23)	-	-
Prescription X	-	-

+ indicates inhibitory spots detected; – indicates no inhibition; Dried water extract re-dissolved in ethyl acetate (W-E); Dried water extract re-dissolved in methanol (W-M); Rhino horn was tested as W-E and W-M only

5.4. Discussion

Currently there is an emergence of drug resistant micro-organisms as a consequence of the increased use of antibiotics (Feucht and Rice, 2003). Natural products research is proving productive in the discovery of new anti-microbials from plant origin (Spitznagel, 1998; Iwu *et al.*, 1999; Xie *et al.*, 2004). An example of an already known anti-microbial compound from TCM herbs is berberine (Iwu *et al.*, 1999). In this study berberine alkaloids were identified in aq. 80% MeOH HPLC-DAD profiles of both Coptidis Rhizoma and *Phellodendron amurense* (Appendix VIIa). Qualitative evaluation of the inhibitory effect of TCM herbs, prescriptions, as well as rhino horn on bacterial growth was conducted. Ethyl acetate extracts of both Coptidis Rhizoma and *Phellodendron amurense* demonstrated anti-bacterial activity against *Bacillus subtilis* in this study.

The results from the anti-bacterial studies show that ethyl acetate extracts of a total of 17 out of the 23 herbs investigated possessed some inhibitory activity against the growth of the Gram-positive bacterium, *Bacillus subtilis* at 100 μg (Table 5.2). The herbs Rhei Radix et Rhizoma and *Salvia miltiorrhiza* also demonstrated inhibitory activity against the Gram-negative bacterium, *Pseudomonas syringae*.

Prescription X (traditionally contains bear bile but was prepared without bear bile in this study) and zhi zi jin hua (composed of eight herbs) did not show any inhibitory activity towards either *B. subtilis* or *P. syringae*. In order to evaluate the contribution of rhino horn (RH) to the anti-bacterial effects of TCMs, ten TCM prescriptions were tested. Five of these prescriptions were composed of rhino horn and herbs, and the other five were made up of only herbs. The results indicate that water extracts (re-dissolved in either ethyl acetate and/or methanol) of QYT, QYTRH, SXD, SXDRH, QGT, QGTRH demonstrated some inhibitory activity against the growth of *B. subtilis* (Table 5.4). Also, SXD and SXDRH possessed some inhibitory activity against the *P. syringae* (Table 5.4). The anti-bacterial tests were qualitative, so although some prescriptions with and without

rhino horn demonstrated anti-bacterial activity, the possible synergistic effect of the horn extracts in the prescriptions could not be assessed. However, water extracts of rhino horn (100 μg) alone did not inhibit the growth of either *B. subtilis* or *P. syringae*. In addition, prescription QGT but not QGTRH demonstrated some inhibitory activity against *P. syringae* (Table 5.4).

Limitations of the method adopted for the anti-bacterial tests in this study include the use of the plant pathogens (*B. subtilis* and *P. syringae*) rather than human pathogens (some of which would require a licence from the Home Office). Also, the technique of direct agar overlay on TLC plate may be more suitable for targeting anti-bacterial agents for local skin application. However, the method has the advantage of enabling the simultaneous testing of a crude extract and groups of compounds from the extract (separated on the TLC plates) and thereby increasing the potential to detect anti-bacterial effects of herbs. Importantly, results from the current study provide evidence to show that fractions showing anti-bacterial activity are likely to possess biological activity. For example, fractions SM6 and SM7 (obtained from *Salvia miltiorrhiza*) inhibited both *B. subtilis* and *P. syringae* and SM8 showed selective strong inhibition towards *B. subtilis* (Table 5.3). When these fractions were tested all three fractions significantly inhibited NF-κB production with SM6 and SM7 showing potent inhibition (Chapter 6; Fig. 6.6). Similarly, fraction SM7 (from *Salvia miltiorrhiza*) demonstrated inhibitory effects in both anti-bacterial (Table 5.2) and cytochrome P450 3A4 (CYP3A4) assays (Chapter 7; Fig. 7.6). This was also demonstrated by fractions 9, 15, 16, and 17 from the qing ying tang prescription and fractions 5 to 12 from *Scutellaria baicalensis*, all of which showed anti-bacterial effects (Tables 5.3 and 5.4) and exhibited inhibitory effects on NF-κB activity, *in vitro* (Chapter 6; Figs. 6.4 and 6.7). Fractions 14 to 20 obtained from fractionating from *Scutellaria baicalensis* showed no apparent anti-bacterial effect on *B. subtilis* and *P. syringae* and also demonstrated no NF-κB inhibitory effects (Fig. 6.3). Furthermore, fraction 14 from *Scutellaria baicalensis* (SB14) with no apparent anti-bacterial effects against *B. subtilis* or *P. syringae* also showed no inhibitory activity towards CYP3A4 activity (Chapter 7; Fig. 7.4). Although there was

some correlation between the anti-bacterial and NF-κB results obtained for the herbal fractions, no definite pattern could be established for the crude extracts of herbs tested. These results are significant because it gives an indication that the anti-bacterial method used in this study could be used to target potentially active pharmacological herbal fractions and subsequently, active compounds.

Recently, Xie et al. (2004) have reported the isolation and identification of lipophilic anti-bacterial compounds from TCM herbs through activity guided fractionations using the TLC based direct agar overlay bioautography technique. Replicating the results obtained from this study, they reported that ethyl acetate herbal extracts demonstrated more anti-bacterial activity compared to aqueous methanol extracts (Xie *et al.*, 2004). Out of the isolated compounds tested, esculetin, a coumarin derivative, demonstrated comparatively stronger inhibitory activity against *P. syringae* at 50-200 µg/ml (Xie *et al.*, 2004).

5.5. Conclusion

Extracts of six out of the seven herbs investigated as herbal alternatives to bear bile showed potential anti-bacterial effects. The herbs were *Anemarrhena asphodeloides*, *Gardenia jasminoides*, *Scutellaria baicalensis*, *Phellodendron amurense*, Coptidis Rhizoma and Rhei Radix et Rhizoma. The anti-bacterial activity of *Andrographis paniculata* was not confirmed in this study. However, some TCM literature cites studies conducted in China, which have shown that *Andrographis paniculata* inhibits several Gram-negative and Gram-positive bacteria *in vitro*, but does not state the concentrations tested (Hsu *et al.*, 1986; Chang and But, 1987; Huang, 1999).

In the investigation of herbal alternatives to rhino horn, extracts of seven herbs showed potential anti-bacterial activity. The herbs were *Salvia miltiorrhiza*, Isatidis Radix, *Rehmannia glutinosa*, *Scrophularia ningpoensis*, Arnebiae seu Lithospermi Radix, *Forsythia suspensa* and *Lonicera japonica*.

Paeoniae Rubra Radix did not demonstrate anti-bacterial activity in this study. The anti-bacterial effects of the herbs indicate potential pharmacological properties. The possible mechanism of anti-inflammatory action of the herbs investigated using nuclear factor kappaB assay is discussed in Chapter 6.

CHAPTER 6. THE EFFECTS OF TRADITIONAL MEDICINES ON NUCLEAR FACTOR KAPPAB ACTIVITY

6.1 Introduction

The overproduction of pro-inflammatory chemicals has been linked to several conditions such as fever (Ivanov and Romanovsky 2004), liver diseases (Tanasescu, 2004), cancer (Ross *et al*., 2004) and cardiovascular diseases (Brown and Jones, 2004). In recent years, the anti-inflammatory mediator nuclear factor-kappaB (NF-κB) has become a therapeutic target for the discovery of novel anti-inflammatory agents from natural products (Bremner and Heinrich, 2002; Calixto *et al*., 2003). NF-κB is induced in response to infections and inflammation and plays a role in almost every aspect of cell regulation including stress responses, immune cell activation, programmed cell death (apoptosis), proliferation control, differentiation, regulation of neuronal survival and tumorigenesis (Luque and Gelinas, 1997; Makarov, 2000; Koulich *et al*., 2001). NF-κB is involved in the production of several pro-inflammatory chemicals including cytokines such as interleukin-6 (IL-6). IL-6 (also known as interferon-β2) is released by tissue macrophages and is a very important pro-inflammatory cytokine and mediator of fever at the level of the central nervous system (Moltz, 1993). It performs multiple functions including the activation of the acute phase reaction. The functions of some other chemical mediators of inflammation regulated by NF-κB are summarised in Table 6.1.

This study was designed to investigate herbal alternatives to bear bile and rhino horn, which are used in the treatment fever and inflammatory conditions in traditional Chinese medicine (TCM). Preliminary studies were conducted to evaluate the effects of selected herbal extracts, fractions and compounds on NF-κB activity as assessed by *in vitro* IL-6 promoter activity.

Table 6.1. Some chemical mediators of inflammation regulated by NF-κB

Chemical mediators	Functions
Lipopolysaccharide (LPS)	LPS (endogenous pyrogen) is released from the cell wall of Gram-negative bacteria during pathogenic infection (Moltz, 1993). LPS can stimulate monocytes and neutrophils to synthesize pro-inflammatory cytokines as well as act directly on the hypothalamus of the brain to elicit fever (Dinarello *et al.*, 1999).
Tumor-necrosis factor-alpha (TNF-α, cachectin)	TNF-α is released during the early phase of the inflammatory response by mast cells to sites of the inflammation where it activates the complement system and further synthesis of its own production. It also induces nitric oxide, IL-1, IL-6, IL-8 and PGE_2 production (Choi, *et al.*, 2001; Dinarello *et al.*, 1999). TNF-α has multiple functions as both a pro-inflammatory and antipyretic agent.
Interleukin-1 (IL-1)	IL-1β is a pro-inflammatory cytokine released by tissue macrophages and functions by causing the enhancement of vascular permeability and the sticking of neutrophils to capillary endothelia as well as coagulation; it also induces B-lymphoctes to divide and mature into antibody producing cells (Spitznagel, 1999). IL-1β can act directly on the brain to cause fever (Dinarello *et al.*, 1999). The combination of IL-1 and TNF-α induces the production of IL-6 (Dinarello *et al.*, 1999). IL-1β also induces the gene expression of COX and IL-8 as well as more production of itself (Dinarello *et al.*, 1999).
IL-8	IL-8 is a powerful chemotaxin whose release from mononuclear phagocytes and endothelial cells is mediated by IL-1 and TNF production and often results in IL-8 being released (Dinarello *et al.*, 1999; Spitznagel, 1999).
IL-12	IL-12 affects the production of TNF (Dinarello, 1997). It stimulates the production of natural killer cells, which secrete γ-interferon leading to the enhancement of the ability of macrophages to kill intracellular organisms (Spitznagel, 1999).
Cycloxygenase-2 (COX-2)	COX catalyses the metabolism of arachidonic acid to prostaglandin H (PGH). PGH is a precursor for the formation of stable prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2) and thromboxane A_2, and prostacyclins (Williamson *et al.*, 1996). Although COX products are induced by IL-6, IL-6 does not affect the gene expression of COX (Dinarello *et al.*, 1999).
Prostaglandin E2 (PGE_2)	PGE_2 induces the production of cyclic AMP, which acts on the hypothalamus of the brain to elicit fever (Kozak *et al.*, 2000). Blocking COX by NSAIDs suppresses the production of PGE_2 which in turn suppresses inflammation and fever (Moltz, 1993).
Inducible nitric oxide synthase (iNOS)	iNOS catalyses the synthesis of nitric oxide (NO) from L-arginine, involving NADPH and molecular oxygen. NO is produced in macrophages as a result of activation by γ-interferon and TNF-α (Spitznagel, 1999) and it in turn induces more production of COX, TNF-α and ROS (Surh *et al.*, 2001). Its functions include vasodilation, cytotoxicity, the killing of microorganisms, inflammation and neurotransmission (Choi *et al.*, 2001).
Reactive oxygen species (ROS)	ROS include free radicals and oxygen ions (Spitznagel, 1999), produced by mechanisms including the respiratory chain, ionizing radiation, NADPH oxidases and myeloperoxidase. ROS can activate NF-κB (Bhattacharyya *et al.*, 2004), as well as cause damage to cell structures and other molecules. ROS also possess essential biological functions including the production of thyroxine and the killing of bacteria by phagocytosis.

6.2. Nuclear Factor-KappaB and the Inflammatory Response

NF-κB is the name for a family of structurally-related DNA-binding proteins that share a highly conserved Rel homology domain (RHD) and recognize a common sequence motif (Karin and Ben-Neriah, 2000). They are transcriptional factors and therefore turn specific genes on or off in response to intra- and extra-cellular signals (Gomez *et al.*, 1997).

Currently there are five identified members of the NF-κB family: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (May and Ghosh, 1997). The N-terminals of the RHD of RelA (p65), RelB, and c-Rel contain the DNA-binding domain required for the dimerization of the NF-κB subunits and subsequent binding to DNA. With the exception of RelB, members of the NF-κB can form homodimers or heterodimers (Tak and Firestein, 2001). The p50/RelA(p65) heterodimer is the major Rel/NF-κB complex in most human cells (May and Ghosh, 1997).

In unstimulated cells, NF-κB resides in the cytoplasm bound non-covalently to a family of inhibitory proteins known as inhibitory-κB (I-κB) (Baldwin, 1996). Currently there are seven known I-κBs: I-κBα, I-κBβ, I-κBγ, I-κBε, Bcl-3, p100 and p105α containing the same ankyrin repeats (May and Ghosh, 1998). The ankyrin repeats interact with a region in the C-terminal domain of the RHD of RelA (p65), RelB, or c-Rel to mask their nuclear localization signal and therefore prevent Rel/NF-κB complexes from being translocated to the nucleus (May and Ghosh, 1998).

Signalling chemicals from the extracellular fluid such as viral toxins (e.g. HIV-1, HTLV-I, HBV, EBV, Herpes simplex), bacterial toxins (LPS, exotoxin B), lymphokines, cytokines (e.g. IL-6, IL-1, TNF-α), nitric oxide, phorbol esters (e.g. phorbol myristate acetate), hepatocyte growth factors, reactive oxygen species (ROS) as well as UV light initiate a cascade of intracellular events leading to the activation of NF-κB (Baldwin, 1996; Gomez *et al.*, 1997). It has been confirmed that the activation of NF-κB requires the phosphorylation of I-κB in serine residues of the N-terminal domain by I-κB

kinases, IKK-α/β/γ (Anest *et al.*, 2003; Yamamoto *et al.*, 2003). This is followed immediately by covalent modification via conjugation with multiple ubiquitin proteins catalysed by ubiquitin ligase complexes in a process known as ubiquitination. This process leads to the rapid degradation of I-κB by the multicatalytic ATP-dependent 26S proteasome complex (Gomez *et al.*, 1997). The complete proteolytic degradation of I-κB results in the dissociation of NF-κB from the I-κB/NF-κB complex (Gomez *et al.*, 1997). The free dimeric NF-κB is quickly translocated into the nucleus where it becomes acetylated and no longer affected by the inhibitory effect of I-κB (Gomez *et al.*, 1997; May and Ghosh, 1997). It then binds to the promoter and/or enhancer regions of a large number of target genes and coordinates their transcription in concert with other transcription factors such as AP1, c-Jun or Sp1 (Tak and Firestein, 2001). NF-κB regulates the expression of genes encoding for chemicals such as IL-6, IL-1 (alpha and beta), TNF-α, COX-2 and iNOS; chemotactants (e.g. IL-8 and monocyte chemoattractant protein 1), cell adhesion molecules (e.g. ICAM-1 and VCAM-1, E-selectin), proliferation signals (e.g. colony-stimulating factor), as well as certain growth and transcription factors (e.g. c-myc, p105, ras and p53) (Gomez *et al.*, 1997; Choi, *et al.*, 2001; Bremner and Heinrich, 2002). Also in a self-regulatory way, the expression of genes for I-κB is encoded by NF-κB (May and Ghosh, 1997). In the nucleus I-κB binds to non-acetylated NF-κB and transports it back to the cytoplasm (May and Ghosh, 1998). A simplified schematic diagram of the NF-κB signalling pathway is given in Fig. 6.1.

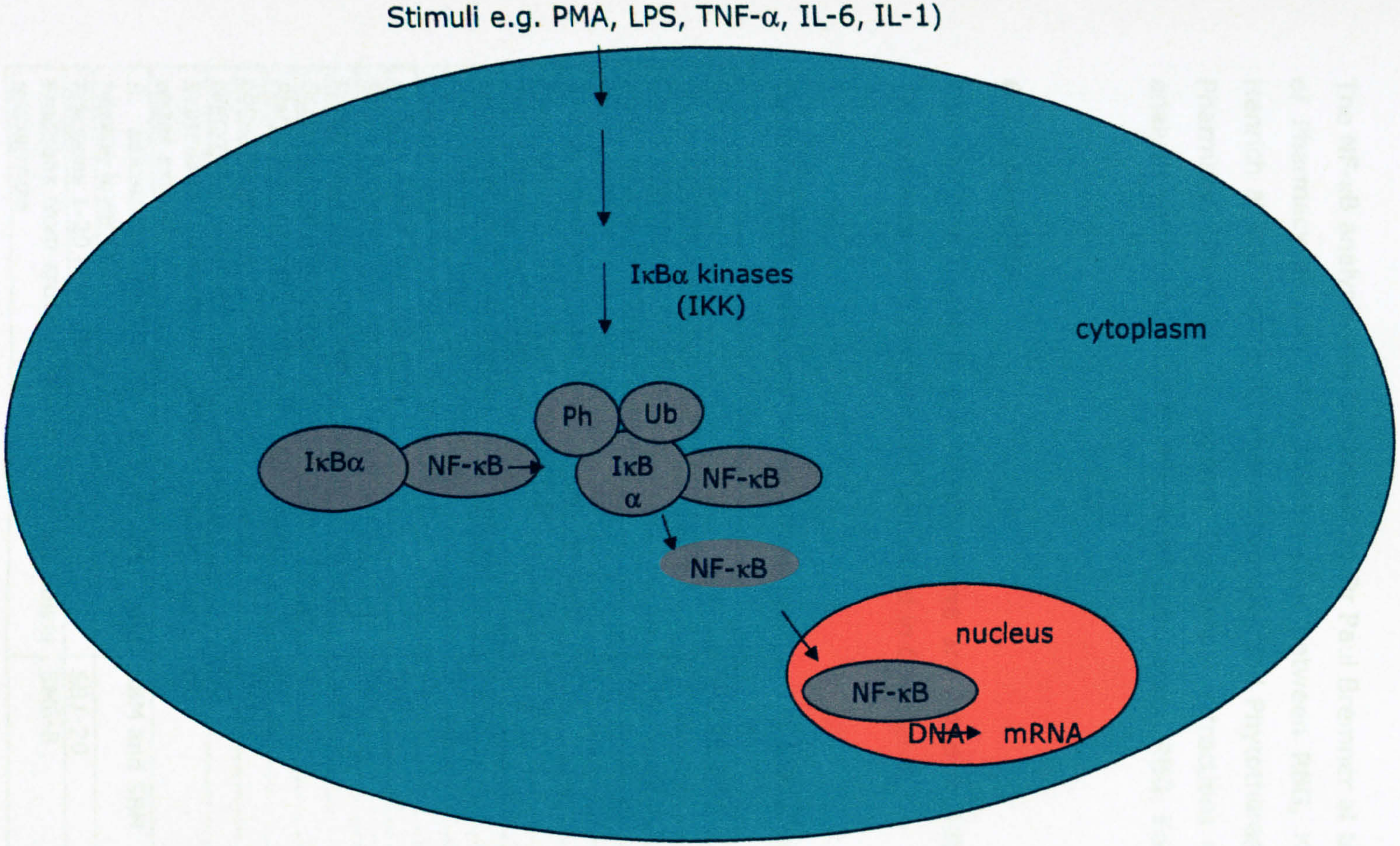


Fig. 6.1. Simplified schematic representation of the nuclear factor kappaB (NF-κB) signalling pathway.
NF-κB activation is stimulated by several extracellular signals which bind to specific membrane receptors and initiate a cascade of events, all converging at the point of IκB kinases (IKK) catalysed phosphorylation of the inhibitory protein I-κB. Phosphorylation (Ph) is followed by poly-ubiquitination (Ub) and rapid proteolytic degradation of I-κB. The free NF-κB dimer is translocated to the nucleus where it participates in the expression of several genes, e.g. for IL-6, IL-1α/β, TNF-α, COX-2, iNOS, IL-8, ICAM-1 and VCAM-1, E-selectin, proliferation signals, growth and transcription factors.

6.3. Experimental Procedure

The NF-κB analysis was conducted by Dr Paul Bremner at the London School of Pharmacy as part of a collaboration between RBG, Kew and Prof. M. Henrich at the Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London. The herbal extractions and the chemical analysis were conducted at the Jodrell Laboratory, RBG, Kew.

6.3.1 Samples

The samples tested in the NF-κB assays are listed in Tables 6.2 and 6.3. The extracts were prepared as described in Chapter 4.

Table 6.2 TCM herbs and prescriptions analysed in the NF-κB assay

Sample	Abbreviation	Graph
Phellodendron amurense Rupr. (Huang bai)	HB	Fig. 6.2
Coptis Rhizoma (huang lian)	HL	Fig. 6.2
Rhei Radix et Rhizoma (Da huang)	DH	Fig. 6.2
Anemarrhena asphodeloides Bge. (zhi mu)	ZM	Fig. 6.2
Andrographis paniculata Nees (chuan xin lian)	CXL	Fig. 6.2
Paeonia suffruticosa Andr. (mu dan pi)	MDP	Fig. 6.3
Trichosanthis Radix (Tian hua fen)	THF	Fig. 6.3
Lophatherum gracile Brongn. (Dan zhu ye)	DZY	Fig. 6.3
Acori Graminei Rhizoma (Chang pu)	CP	Fig. 6.3
Paeonia lactiflora Pall. (chi shao yao)	CSY	Fig. 6.3
Isatidis Radix (ban lan gen)	BLG	Fig. 6.3
Sojae Praeparatum semen (Dan dou chi)	DDC	Fig. 6.3
Rehmannia glutinosa Steud. (sheng di huang)	SDH	Fig. 6.3
Scrophularia ningpoensis Hemsl. (xuan shen)	XS	Fig. 6.3
Platycodon grandiflorum A.DC. (jie geng)	JG	Fig. 6.3
Lonicera japonica Thunb. (jin yin hua)	JYH	Fig. 6.3
Glycyrrhiza uralensis Fisch. (gan cao)	GC	Fig. 6.3
Scutellaria baicalensis Georgi (huang qin), water extract	HQ	Fig. 6.2
S. baicalensis methanolic extract (M) and hexane layer (H)	SBM and SBH	Fig. 6.4
Fractions 1-20 from SBM	SB1-20	Fig. 6.4
Fractions from crude methanolic extract *Salvia miltiorrhiza*	SM6-8	Fig. 6.7

Table 6.2 cont'd.

Sample	Abbreviation	Graph
Rhino horn (Xi jiao) (RH)	RH	Fig. 6.6
Zhi zi jin hua	ZZJH	Fig. 6.6
Xi jiao dihuang tang without RH	XJDHT	Fig. 6.6
Xi jiao dihuang tang with RH	XJDHTRH	Fig. 6.6
Sheng xi dan without RH	SXD	Fig. 6.6
Sheng xi dan with RH	SXDRH	Fig. 6.6
Qing gong tang without RH	QGT	Fig. 6.6
Qing gong tang with RH	QGTRH	Fig. 6.6
Qingwen baidu yin without RH	QWBY	Fig. 6.6
Qingwen baidu yin with RH	QWBYRH	Fig. 6.6
Qing ying tang without RH	QYT	Fig. 6.6
Qing ying tang with RH	QYTRH	Fig. 6.6
Fractions from QYT	QYT9, QYT15, QYT16, QYT17	Fig. 6.7

Table 6.3. The final concentrations of compounds tested in the NF-κB assay

Compounds and molecular weight	Concentration µg/ml	Concentration µM
Chrysin 5,7-dihydroxyflavone (254)	100 25.4 12.7 6.4	393 100 50 25
Baicalein 5,6,7-trihydroxyflavone (270)	100 27.0 13.5 6.8	370 100 50 25
Wogonin 5,7-dihydroxy-8-methoxyflavone (284)	28.4 14.2 7.1	100 50 25
Oroxylin A 5,7-dihydroxy-6-methoxyflavone (284)	100 28.4 14.2 7.1	352 100 50 25
Scutellarein 5,6,7,4'-tetrahydroxyflavone (288)	100	347
Baicalin 5,7-dihydroxy-7-glucuronide (446)	100	224
Salicylic acid 2-hydroxybenzoic acid (138)	20	145

6.3.2. NF-κB studies using the IL-6 promotor assay method

The assay used was as described by Vasilev et al. (2005). HeLa S3 cells were stably transfected with IL-6 promoter fused with a Luciferase reporter

gene for 24 hours. Extracts/fractions (final incubation concentration of 100 μg/ml in DMSO; Table 6.2) and compounds (prepared in acetone; Table 6.3) were placed in 96-well plates and incubated with the transfected cells (at 37 ^{O}C; 95% humidity) for 1 hour. The cells were then stimulated with 50 ng/ml (final concentration) of either phorbol myristate acetate (PMA, Sigma, UK) or TNF-α (Sigma, UK) and incubated for a minimum of 7 hours (maximum 14 hours) before harvesting. To each well, 100 μl of lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 7% glycerol) was added and left for 15 minutes. After harvesting the cells, beetle Luciferin (50 μl, Promega, USA) was added to the lysed cells (15 μl) in a 96-well plate by an automated Luminoter/photometer (Anthos Lucy 1, Rosys Anthos, Switzerland) and the light emission was measured following a reaction time of 10 seconds. The light emission of the lysis buffer was obtained as a background reading and subtracted from each experimental value. Positive controls consisted of cells stimulated with either PMA or TNF-α only and negative controls involved cells subjected to no stimulation.

6.3.3. Data analysis

Results were obtained as the light emission (Luciferase values) from three replicates. Measurements from resting cells, representing minimal production of NF-κB (as indicated by minimal IL-6 promoter activity) were used as negative controls. The average Luciferase value of the positive controls (cells stimulated with TNF-α or PMA only) was assumed to be equivalent to 100% production of NF-κB. Therefore, NF-κB activity was calculated as a percentage of the positive controls. The values obtained were expressed as mean values ± SD. The Student's 1-sample *t*-test was used to determine statistical differences between test and positive control groups. The difference was considered statistically significant when $p<0.05$.

6.4. Results

6.4.1. The effects of individual herbs on NF-κB activity

Crude hot water extracts of 18 herbs were tested in the NF-κB assay. Compared to the water extracts of the other herbs, the most potent inhibition of NF-κB activity was obtained for *Paeonia suffruticosa* (MDP) and Trichosanthis Radix (THF) which significantly reduced NF-κB activity by 56% ($p<0.001$) and 50% ($p<0.01$), respectively (Fig. 6.2). Rhei Radix Rhizoma (DH) (Fig. 6.3), *Lophatherum gracile* (DZY), Isatidis Radix (BLG), Sojae Praeparatum Semen (DDC) and *Rehmannia glutinosa* (SDH) (Fig 6.2) also showed significant ($p<0.05$) inhibition of NF-κB activity. *Phellodendron amurense* (huang bai; HB) and Coptidis Rhizoma (HL) reduced NF-κB activity by 35% and 27%, respectively (Fig. 6.3); in addition, reductions of 38% and 34% were obtained for extracts of Acori Graminei Rhizoma (CP) and *Paeonia lactiflora* (CSY), respectively (Fig 6.2). However, these reductions were not statistically significant.

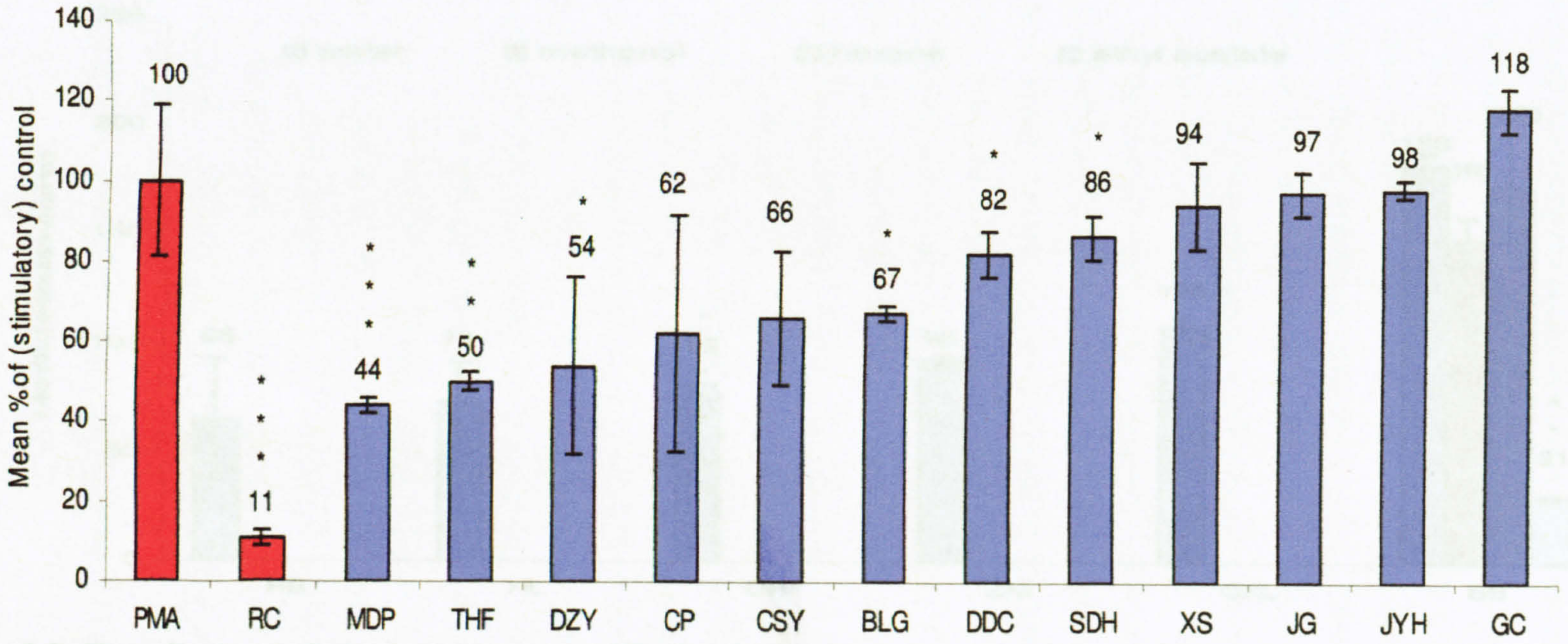


Fig. 6.2. The effects of 12 herbal extracts on NF-κB activity.
Crude hot water (100 μg/ml) were tested in PMA-stimulated cells as outlined in Section 6.32.2. Resting cells (RC) were used as negative controls; cells stimulated with PMA alone were used as positive controls. The data represent mean (n = 7 for controls and n = 3 for test samples) ± SD. *p< 0.05, **p<0.01, ***p<0.001 indicate statistically significant differences from groups only treated with PMA. The full names of the herbs are described in Table 6.2.

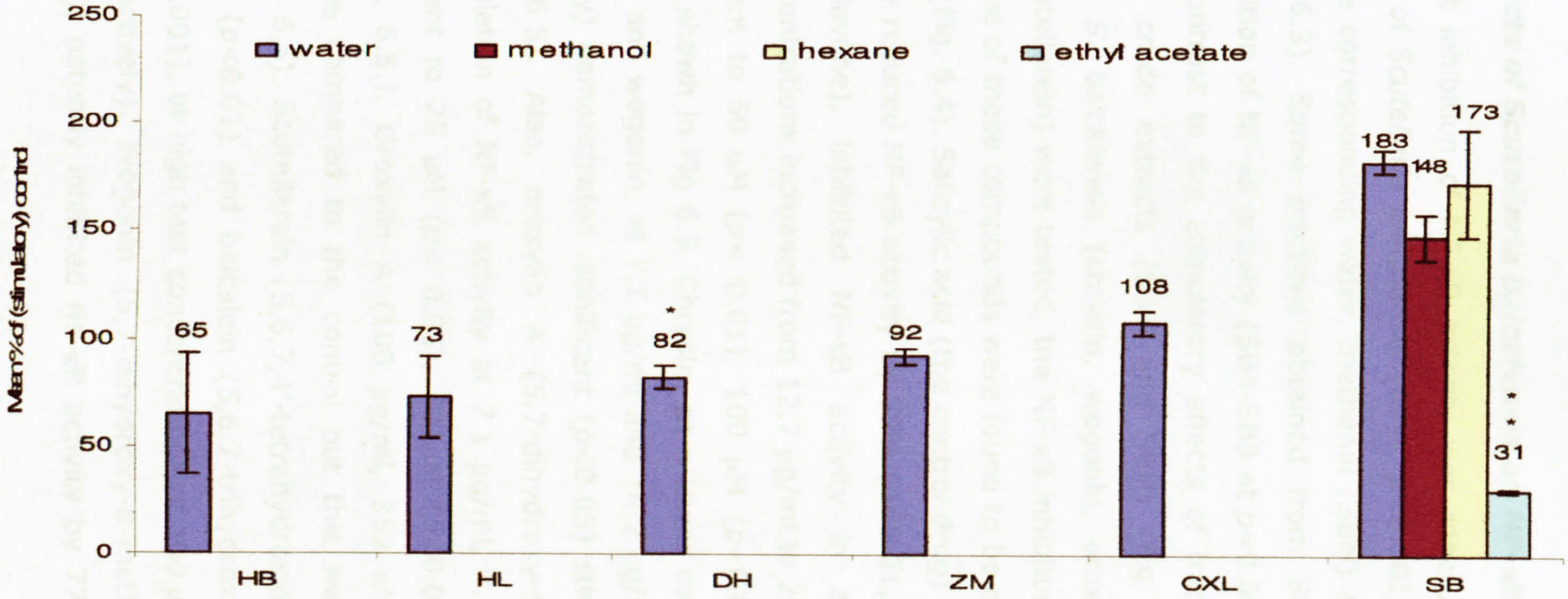


Fig. 6.3. The effects of six herbal extracts on NF-κB activity.
Cells for water extracts were stimulated with phorbol myristate acetate (PMA; 50 ng/ml) and the ethyl acetate extract with TNF-α (50 ng/ml). Induced IL-6 promoter activity gene transcription was measured as light emission (Luciferase values) expressed as a percentage relative to cells stimulated by PMA or TNF-α only. The data represent mean (n = 3) ± SD. *p<0.05 and **p<0.001 indicate statistically significant differences from cells treated with PMA or TNF-α only.
HB = huang bai, *Phellodendron amurense* Rupr; HL = huang lian, Coptidis Rhizoma; ZM = zhi mu, *Anemarrhena asphodeloides* Bge; CXL = chuan xin lian, *Andrographis paniculata* Nees; SB = *Scutellaria baicalensis* Georgi.

6.4.2. The effects of Scutellaria baicalensis on NF-κB activity

Although potent inhibition of NF-κB activity was measured for the ethyl acetate extract of *Scutellaria baicalensis* (69%, $p<0.001$), stimulation was obtained for the corresponding water, methanol (SBM) and hexane (SBH) extracts (Fig. 6.3). Some fractions obtained from SBM demonstrated significant inhibition of NF-κB activity (SB4-SB9 at $p=0.001$; SB10-SB13 at $p=0.001$), in contrast to the stimulatory effects of fractions SB2, SB14-SB17 and the crude extracts (SBM and SBH) (Fig. 6.4). When the constituents of *S. baicalensis* (chrysin, wogonin, oroxylin A, baicalein, baicalin and scutellarein) were tested, the NF-κB inhibitory and stimulatory activities of some of these compounds were found to be dependent on their concentrations (Fig. 6.4). Salicylic acid (the control drug) at 100 μg/ml (145 μM) significantly reduced NF-κB activity by 59% ($p<0.01$; Fig. 6.5). Chrysin (5,7-dihydroxyflavone), inhibited NF-κB activity in a dose-dependent manner as concentrations increased from 12.7 μg/ml to 25.4 μg/ml and 100 μg/ml (equivalent to 50 μM ($p< 0.01$), 100 μM ($p=0.001$) and 393 μM ($p=0.001$)), as shown in Fig 6.5. Chrysin, at a lower concentration of 6.4 μg/ml (25 μM), and wogonin, at 7.1 μg/ml and 14.2 μg/ml (25 μM and 50 μM, respectively) demonstrated significant ($p<0.05$) stimulation of NF-κB activity (Fig. 6.5). Also, oroxylin A (5,7-dihydroxy-6-methoxyflavone) caused a stimulation of NF-κB activity at 7.1 μg/ml, 4.2 μg/ml and 28.4 μg/ml (equivalent to 25 μM ($p< 0.05$), 50 μM ($p<0.001$), and 100 μM ($p<0.01$)) (Fig. 6.5.). Oroxylin A (100 μg/ml, 352 μM) reduced NF-κB activity by 14% compared to the control but this was not statistically significant (Fig. 6.5). Scutellarein (5,6,7,4′-tetrahydroxyflavone) showed a 35% reduction ($p<0.01$) and baicalein (5,6,7-trihydroxyflavone), a 90% reduction ($p<0.001$), at high test concentrations of 100 μg/ml (347 μM and 370 μM, respectively). Wogonin (5,7-dihydroxy-8-methoxyflavone; 28.4 μg/ml; 100 μM), potently inhibited NF-κB activity by 77% ($p<0.001$; Fig. 6.5).

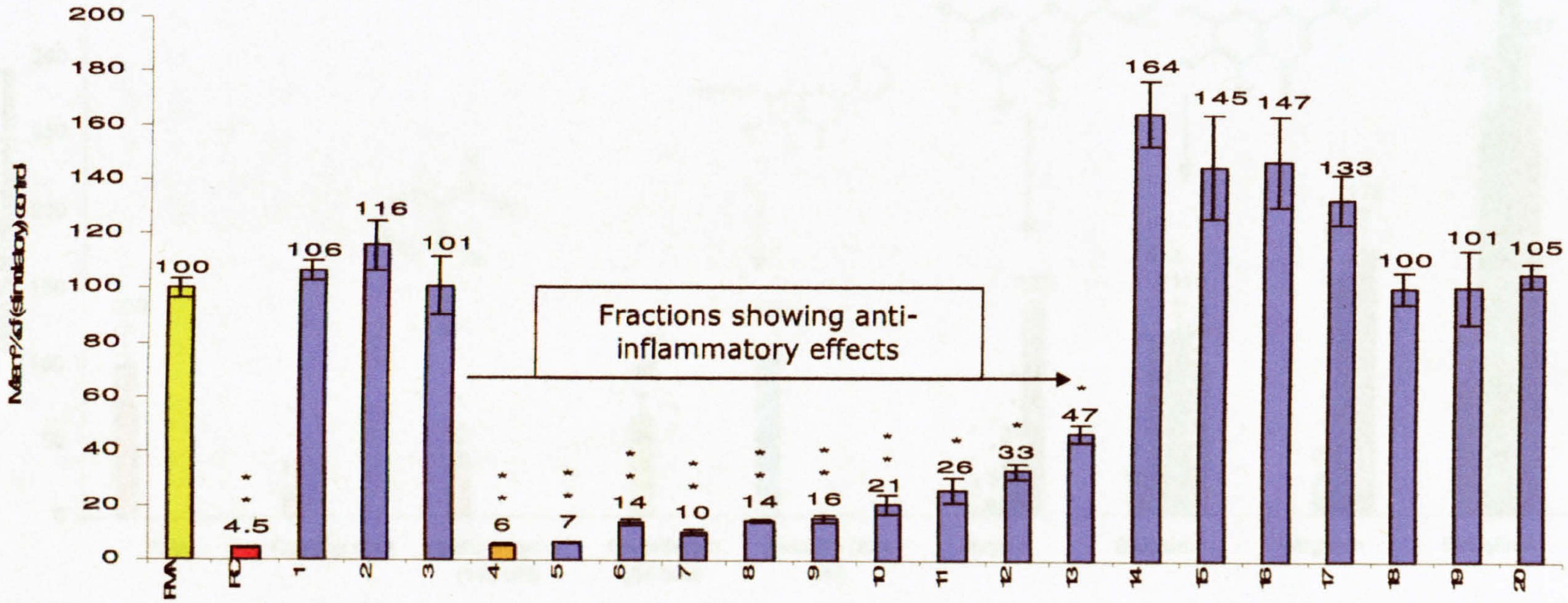


Fig. 6.4. The effects of fractions obtained from methanol extract of *Scutellaria baicalensis* on NF-κB activity.
Fractions (100 μg/ml) were tested in PMA-stimulated cells. The method is outlined in Section 6.32.2. Negative controls: resting cells (RC; unstimulated); positive controls: PMA stimulated cells. The data represent mean (n = 9 for controls and n = 3 for test samples) ± SD. *p= 0.001, **p<0.001 indicate statistically significant differences from PMA-stimulated cell. Colour code: yellow = positive control (stimulatory); red = resting cells (inhibitory); purple = other fractions obtained from fractionating the crude extract; orange = fraction further fractionated (mean % of stimulatory control of Fraction 4v = 3%, $p < 0.001$; Fraction 4vi = 3%, $p < 0.001$; Fraction 4viii = 12%, $p < 0.001$.

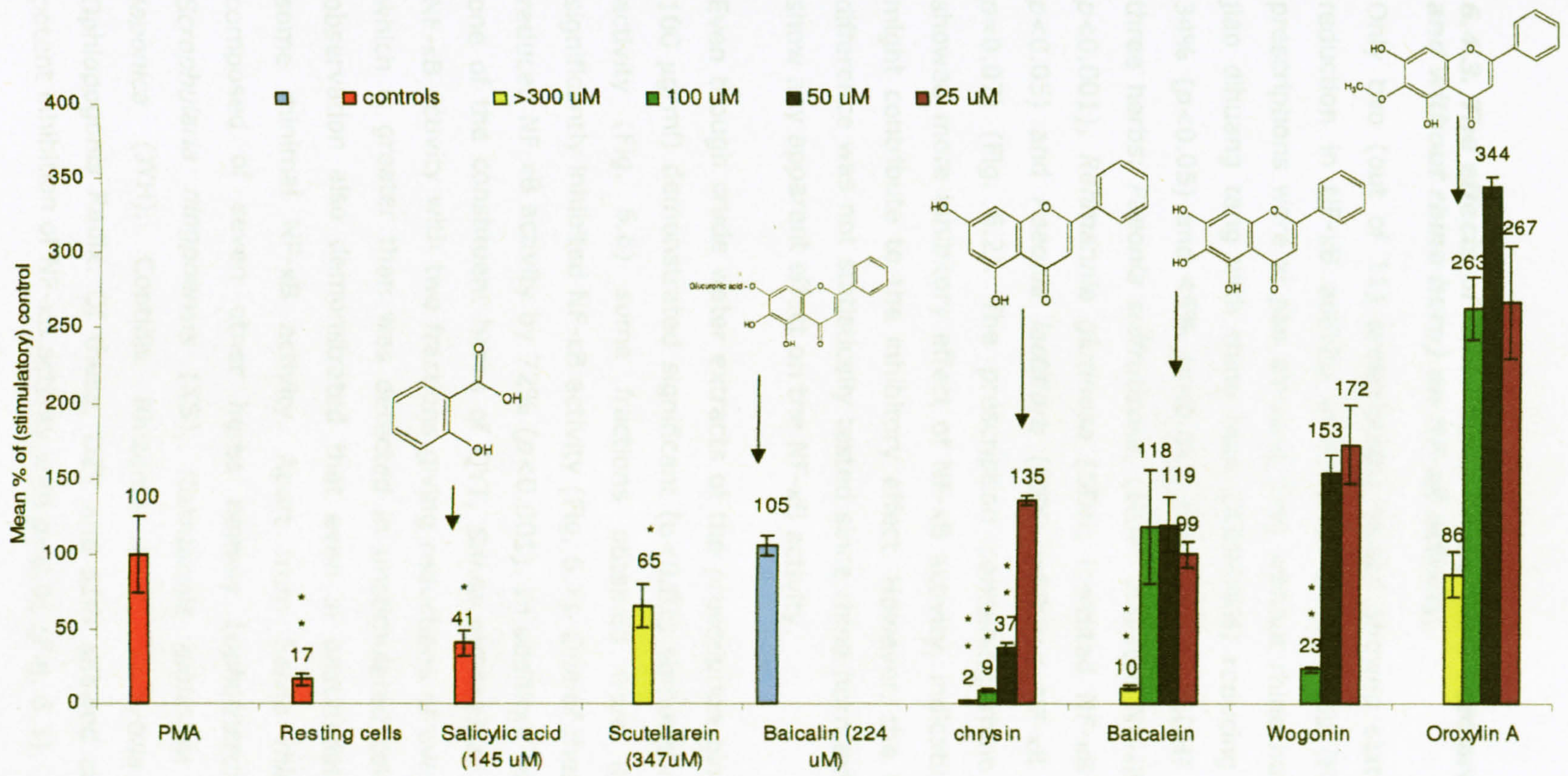


Fig. 6.5. The effects of flavonoids from *Scutellaria baicalensis* and salicyclic acid on NF-κB activity.
Extracts (100 µg/ml) were tested in PMA-stimulated cells. IL-6 genepromoter activity transcription was measured as outlined in Section. 6.32.2. Negative controls: resting cells (unstimulated); positive controls: PMA stimulated cells. The data represent mean (n = 8 for PMA, n = 9 for resting cells and n = 3 for test compounds) ± SD. *p< 0.01, **p<0.001 indicate statistically significant differences from PMA-stimulated cells. Scutellarein (5,6,7,4′-tetrahydroxyflavone); baicalin (5,6-dihydroxy-7-glucuronide); chrysin (5,7-dihydroxyflavone); baicalein (5,6,7-trihydroxyflavone); wogonin (5,7-dihydroxy-8-methoxyflavone); oroxylin A (5,7-dihydroxy-6-methoxyflavone).

6.4.3. The effects of rhino horn extract and TCM prescriptions (with and without rhino horn) on NF-κB activity.

Only two (out of 11) prescriptions tested showed statistically significant reduction in NF-κB activity in PMA-stimulated cells (Fig. 6.6). The two prescriptions were xi jiao dihuang tang without rhino horn (XJDHT) and xi jiao dihuang tang with rhino horn (XJDHTRH) reducing NF-κB activity by 34% ($p<0.05$) and 45% ($p<0.01$), respectively. XJDHT was composed of three herbs: *Paeonia suffruticosa*, (MDP: inhibited NF-κB activity by 66%, $p<0.001$), *Rehmannia glutinosa* (SDH; inhibited NF-κB activity by 14%, $p<0.05$) and *Paeonia lactiflora* (CSY; inhibited NF-κB activity by 34%, $p=0.07$) (Fig. 6.2). The prescription containing rhino horn (XJDHTRH) showed more inhibitory effect of NF-κB activity, indicating that rhino horn might contribute to the inhibitory effect. However, the significance of the difference was not statistically tested since rhino horn extract alone did not show any apparent effect on the NF-κB activity.

Even though crude water extracts of the prescription qing ying tang (QYT; 100 μg/ml) demonstrated significant ($p<0.01$) stimulatory effect on NF-κB activity (Fig. 6.6) some fractions obtained from the crude extract significantly inhibited NF-κB activity (Fig. 6.7). One of these fractions QYT17 reduced NF-κB activity by 72% ($p<0.001$). In addition, some fractions from one of the constituent herbs of QYT, *Salvia miltiorrhiza* potently inhibited NF-κB activity with two fractions giving reductions of over 90% ($p<0.001$), which is greater than was detected in unstimulated cells (Fig. 6.6). This observation also demonstrated that even in unstimulated cells, there is some minimal NF-κB activity. Apart from *Salvia miltiorrhiza*, QYT is composed of seven other herbs namely *Lophatherum gracile* (DZY), *Scrophularia ningpoensis* (XS), *Rehmannia glutinosa* (SDH), *Lonicera japonica* (JYH), Coptidis Rhizoma (HL), *Forsythia suspensa*, and Ophiopogonis Radix. Of these, DZY and SDH showed significant, but not potent inhibition of NF-κB activity with $p<0.05$ (Fig. 6.3).

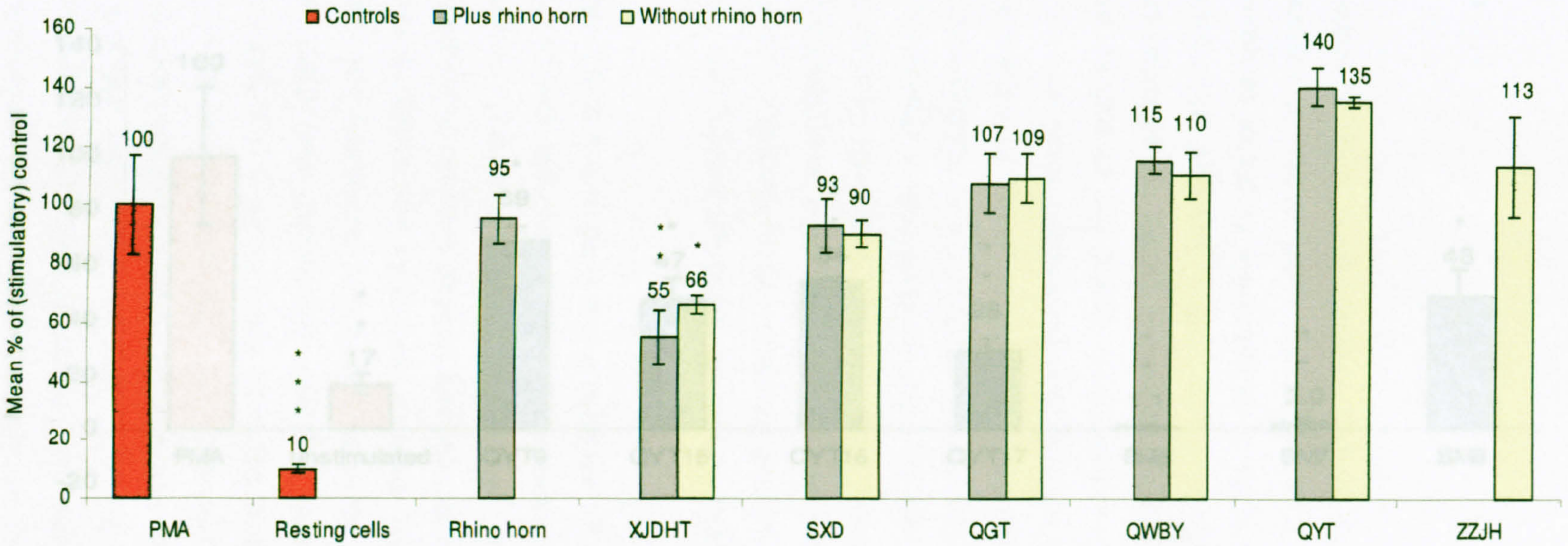


Fig. 6.6. The effects of rhino horn extract and TCM prescriptions (with and without rhino horn) on NF-κB activity.
Water extracts (100 μg/ml) were tested in PMA-stimulated cells as outlined in Section 6.32.2. Resting cells were used as negative controls; cells stimulated with PMA alone were used as positive controls. The data represent mean (n = 6 for PMA, resting cells and rhino horn; and n = 3 for other test samples) ± SD. *$p< 0.05$, **$p<0.01$, ***$p<0.001$ indicate statistically significant differences from groups only treated with PMA. The full names of the prescriptions are described in Table 6.2.

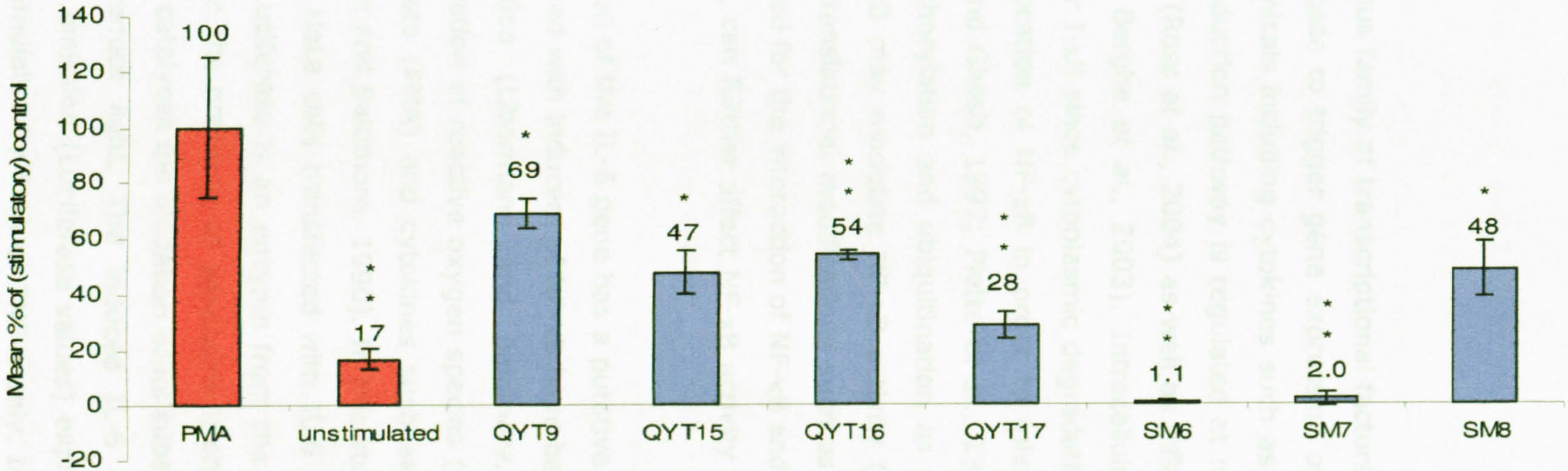


Fig. 6.7. The effects of fractions obtained from a methanol extract of *Salvia miltiorrhiza* and water extract of qing ying tang (GYT) on NF-κB activity.

Extracts (100 μg/ml) were tested in PMA-stimulated cells as outlined in Section 6.32.2. Unstimulated cells were used as negative controls; cells stimulated with PMA alone were used as positive controls. The induced IL-IL-6 promotergene transcription activity was measured as light emission (Luciferase values) and expressed as a percentage relative to cells stimulated with PMA alone. The data represent mean (n = 8 for PMA, n = 9 for resting cells and n = 3 for test samples) ± SD. **p*=0.01, ***p*<0.001 indicate statistically significant differences from groups only treated with PMA.

6.5. Discussion

NF-κB is a ubiquitous family of transcriptional factors that forms part of the cell signalling cascade to trigger gene expression of a wide range of pro-inflammatory chemicals including cytokines such as IL-6 (Table 6.1.). The NF-κB signal transduction pathway is regulated at multiple levels and in a variety of cancers (Ross *et al.*, 2004) as well as inflammatory and immune diseases (Vanden Berghe *et al.*, 2003). Intracellular regulators of NF-κB include its inhibitor I-κB since cytoplasmic degradation of I-κBs is essential for nuclear translocation of NF-κB in order to interact with specific DNA sequences (May and Ghosh, 1997; Piette *et al.*, 1997). Therefore, factors affecting the phoshorylation and ubiquitination as well as the proteolytic degradation of I-κB may modulate NF-κB activity (Vanden Berghe *et al.*, 2003). Also post-translational modifications such as the acetylation of NF-κB, which is required for the interaction of NF-κB and DNA in the nucleus for gene transcription, can further affect NF-κB activity (Vanden Berghe *et al.*, 2003).

The promoter region of the IL-6 gene has a putative NF-κB binding site and HeLa cells stimulated with inducers of NF-κB have been reported to activate IL-6 gene induction (Libermann and Baltimore, 1990). Inducers of endogenous generation of reactive oxygen species (ROS) such phorbol 12-myristate 13-acetate (PMA) and cytokines such as TNF-α induces NF-κB activity (Libermann and Baltimore, 1990). In this study TNF-α or PMA were used to stimulate HeLa cells transfected with IL-6 promoter fused with a Luciferase gene. Luciferase is an enzyme from the North American firefly *Photinus pyralis*. In the presence of adenosine triphosphate and molecular oxygen Luciferase catalyses the oxidation of its substrate beetle luciferin to oxyluciferin to produce light. The induced IL-6 promoter activity was measured as light emission (Luciferase values) expressed as a percentage relative to cells stimulated by PMA or TNF-α only. In the NF-κB studies an anti-inflammatory activity was indicated by inhibition of IL-6 reporter activity.

The NF-κB tests described in this Chapter as well as anti-bacterial tests (described in Chapter 5) were conducted to assess the traditional claims of selected herbs as anti-inflammatory and anti-bacterial agents. *Lophatherum gracile* (DZY), Sojae Praeparatum Semen (DDC), Isatidis Radix (BLG), *Rehmannia glutinosa* (SDH) and Rhei Radix et Rhizoma (DH) showed statistically significant NF-κB inhibition (Figs. 6.2 & 6.3) as well as anti-bacterial activity against the Gram-positive bacterium *Bacillus subtilis* (Table 5.2). To the author's knowledge, this is the first report of the NF-κB activity and anti-bacterial effects of *Lophatherum gracile* and Sojae Praeparatum Semen in the English language. *Rehmannia glutinosa* has been reported to inhibit COX-2, TNF-α and IL-1 secretions, *in vitro* (Kim *et al.*, 1999). The anti-inflammatory effects of compounds isolated from Isatidis Radix (Ishihara *et al.*, 2000; Molina *et al.*, 2001; Danz *et al.*, 2002) as well as from Rhei Radix et Rhizoma (Matsuda *et al.*, 2000; Kageura *et al.*, 2001) have also been reported.

Interestingly, Trichosanthis Radix (THF) and Acori Graminei Rhizoma (CP), showed no apparent inhibitory activity against *B. subtilis* (of the ethyl acetate extract) (Table 5.2) and the water extracts caused some inhibition of NF-κB activity (Fig. 6.2). Trichosanthis Radix potently inhibited NF-κB activity and again to the author's knowledge, this is the first report of the effect of Trichosanthis Radix on NF-κB activity in the English language. However, Osaki et al. (1996) reported that 50% ethanol extracts of the fruit of *Trichosanthes kirilowii* demonstrated anti-inflammatory effects, *in vivo* and Lee et al. (1995) reported that methanol extract (0.05 mg/ml) of the seeds caused significant inhibition of interleukin-8 (IL-8), *in vitro*. Trichosanthis Radix has been reported to possess anti-cancer properties (Dou and Li, 2004; Lee and Wong, 2005) and the results from NF-κB data from this study may indicate that one possible mechanism is via the inhibition of IL-6 promoter activity.

Water extracts of *Paeonia suffruticosa* showed potent NF-κB activity via the inhibition of IL-6 promoter activity in this study (Fig. 6.2). A compound isolated from *Paeonia suffruticosa*, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, has been reported to inhibit IL-8 via NF-κB binding inhibition (Oh *et al.*,

2004) and also inhibit inducible nitric oxide synthase and COX-2 activity (Lee *et al.*, 2003). Water extracts of *Lonicera japonica* (JYH) showed no significant inhibition of NF-κB activity in this study (Fig. 6.2) but it has been reported to inhibit NF-κB through the degradation of I-κBα in the liver of LPS-challenged rats (Lee *et al.*, 2001).

It was also observed during the investigations that fractions from *Scutellaria baicalensis* and *Salvia miltiorrhiza*, which had shown some anti-bacterial properties (Table 5.3) in the current study, also demonstrated anti-inflammatory effects by causing reductions in NF-κB activities (Figs. 6.4 and 6.7). This pattern was also observed for fractions of qing ying tang (Table 5.4 and Fig. 6.7), a TCM formulation, composed of eight herbs. The eight herbs are *Salvia miltiorrhiza*, *Scrophularia ningpoensis*, *Rehmannia gluitinosa*, *Lonicera japonica*, *Forsythia suspense*, Coptidis Rhizoma, Ophiopogonis Radix and *Lophatherum gracile*.

Data obtained for *S. baicalensis* showed that the methanol extract that caused stimulation of NF-κB activity was composed of different components; some possessing potent stimulatory activity, some with potent inhibitory activity and some with a very marginal effect on the activity of NF-κB (Fig. 6.4). This effect was also demonstrated by the flavonoid compounds (oroxylin A, chrysin and wogonin) isolated from *S. baicalensis* in this study, which all caused inhibition of NF-κB at certain concentrations and stimulation at lower concentrations (Fig. 6.5). However, the NF-κB inhibition by oroxylin A (5,7-dihydroxy-6-methoxyflavone; 352 μM) in HeLa cells stimulated by PMA was not statistically significant. In LPS-induced NF-κB activity in RAW264.7 macrophages, oroxylin A (70 μM) has been reported to cause inhibition of the NF-κB complex (Chen *et al.*, 2000). The inhibitory NF-κB activities of wogonin (5,7-dihydroxy-8-methoxyflavone), chrysin (5,7-dihydroxyflavone) as well as for baicalein (5,6,7-trihydroxyflavone) and scutellarein (5,6,7,4′-tetrahydroxyflavone) were demonstrated in the present study (Fig. 6.5). Recently, baicalein has been reported to potently inhibit IL-12 production in LPS-activated macrophages via the inhibition of NF-κB binding activity at concentrations of 24 μM, 48 μM and 96 μM (Kang *et al.*, 2003a). Both baicalein and wogonin have also been reported to

inhibit IL-1β-induced IL-6 and IL-8 mRNA encoded by NF-κB (Nakamura *et al.*, 2003). It was also reported that wogonin, but not baicalein suppressed NF-κB binding activities (Nakamura *et al.*, 2003). In the current study, baicalin (5,6-dihydroxy-7-glucuronide) had little apparent inhibitory effect on NF-κB activity which is consistent with reports that baicalin does not affect NF-κB activity (Nakamura *et al.*, 2003; Chen *et al.*, 2004). However, a recent report based on studies conducted in China stated that baicalin may inhibit NF-κB activity through the inhibition IκB-α expression (Yang *et al.*, 2005). Anti-oxidants (such as flavonoids) can modulate NF-κB activity through the inhibition of reactive oxygen species (Bhattacharyya *et al.*, 2004) and this is therefore one possible mechanism of action for the compounds studied.

Original investigations were also conducted to assess the contribution of rhino horn extract to possible anti-inflammatory effects of TCM prescriptions as assessed by NF-κB inhibitory activity. Water extracts of rhino horn did not demonstrate NF-κB inhibitory activity. However, the TCM prescriptions xi jiao dihuang tang (XJDHT) and XJDHT containing rhino horn showed significant inhibition of NF-κB activity (Fig. 6. 7). Since the contribution of the horn extracts to the NF-κB inhibitory activity of the TCM prescriptions could not be ascertained further work using other bioassays may be required to determine possible synergistic effects. Fractions of prescriptions qing ying tang (QYT; without the animal product) also demonstrated NF-κB inhibitory activities (Fig. 6. 7).

6.6. Conclusions

Two out of the six herbs selected for study as possible replacements for bear bile, (namely Rhei Radix Rhizoma and *Scutellaria baicalensis*) demonstrated anti-inflammatory properties through the inhibition of NF-κB activity. In addition, inhibitory NF-κB activity was demonstrated for four out of the nine herbs selected for study as possible replacements for rhino horn: *Paeonia suffruticosa*, Isatidis Radix, *Rehmannia glutinosa* and *Salvia*

miltiorrhiza. Trichosanthis Radix, Sojae Praeparatum Semen and *Lophatherum gracile*, which are found in different prescriptions, also demonstrated inhibitory NF-κB activity. To date no scientific literature in the English language has reported the inhibitory NF-κB effects of Trichosanthis Radix, Sojae Praeparatum Semen and *Lophatherum gracile*. Therefore, further studies are required to verify the data obtained from this study. Eleven prescriptions were investigated, one prescription containing rhino horn extract and two prescriptions composed of only herbs showed inhibitory NF-κB activity. Based on these preliminary results it can be proposed that some TCM prescriptions in the absence of rhino horn could still be efficacious as anti-inflammatory agents.

The herbs proposed as alternatives to animal products (Chapter 8) are often combined in TCM prescriptions and/or co-medicated with Western pharmaceutical drugs. Therefore, possible interactions between drug-herb interactions were also investigated and the results are described in Chapter 7.

CHAPTER 7. CYTOCHROME P450 INHIBITION STUDIES

7.1. Introduction

There is an increase in the use of herbal medicines both in the developing and industrialized countries (WHO, 2004). Rather than as an alternative to Western pharmaceutical drugs, herbal medicine is often used in conjunction with them (Wadsworth *et al.*, 2003; Harrison *et al.*, 2004). Herbal and pharmaceutical drugs are sometimes co-administered for perceived synergistic or complementary effects. Some traditional Chinese medicine (TCM) consumers also use herbal medicine with prescribed pharmaceutical drugs to counteract the perceived side effects of the pharmaceutical drugs (Thai and Owen, 2003). Users of herbal medicines often fail to inform their doctor or pharmacist when prescribed a pharmaceutical drug and doctors often do not enquire (WHO, 2002). Currently there is a lack of substantial research data on the majority of herbs used as medicines or consumed in foods, to help make informed decisions about the compatibility of these herbs with pharmaceutical drugs. However, the study of herbs on drug metabolising enzymes is a growing area of research. For example, studies on some commonly used Western herbs such as *Hypericum perforatum* (St John's Wort) (Zou *et al.*, 2002, Markowitz *et al.*, 2003), *Gingko bilobae* L. (Zou *et al.*, 2002), *Oenothera biennis* (evening primrose oil) (Zou *et al.*, 2002), *Alium savitum* (garlic) (Zou *et al.*, 2002;, Zhou *et al.*, 2003), *Piper methysticum* (kava kava root) (Zou *et al.*, 2002, Unger and Frank, 2004), *Harpagophytum procumbens* (devil's claw) (Unger and Frank, 2004) and *Tanacetum parthenium* (feverfew herb) (Unger and Frank, 2004) have highlighted the potential of herbs and phytochemicals to change the pharmacodynamic and pharmacokinetic properties of drugs through the modulation of cytochrome P450s.

In the current research, TCM herbs are investigated as alternatives to animal products (bear bile and rhino horn). The quality of the herbs was

assessed (Chapter 3) and the efficacy of the herbs as anti-bacterial (Chapter 5) and their effect on nuclear factor kappaB activity (Chapter 6) were also studied. This Chapter addresses safety issues relating to combination of medicinal products to form prescriptions and the co-medication of herbs/presciptions with Western pharmaceutical medicines which may lead to drug-herb interactions. This investigation is important since the herbs proposed as alternatives to the animal products (Chapter 8) are more likely to be used in combination with other herbs rather than singly. Therefore, as part of this study, cytochrome P450 assays were conducted and a novel assay was developed to simultaneously investigate possible drug-herb interactions based on a group of CYP enzymes and to monitor the effects of the herbs on the production biologically active eicosanoids.

7.2. Cytochrome P450

Cytochrome P450s represent a gene superfamily of membrane-bound, heme-containing terminal oxidases (El-Sankary *et al.*, 2001). They were first identified by their unique spectral properties with a near-UV λmax at 450 nm for the Fe^{II}-CO complex (Klingenberg, 1958; Omura and Sato, 1964). Currently in humans, 57 sequenced (functional) genes and 58 pseudogenes (a gene relic that is not expressed in normal tissue) are known and divided into 17 families (Nelson *et al.*, 2004). The CYPs are constitutively expressed principally in the liver as well as in lower amounts in most cell types, except erythrocytes and striated muscle (Guengerich and Shimada, 1991; Chen *et al.*, 2001a).

CYPs are responsible for the degradative metabolism of hydrophobic endogenous substances (endobiotics) and metabolism of foreign compounds (xenobiotics) including drugs, phytochemicals, carcinogens and pesticides into non-toxic forms and in some cases biologically active or toxic forms (Guengerich, 1989; Tesfaigzi *et al.*, 2001). Also, CYPs are involved in the metabolism of endogenous steroid hormones, fat-soluble vitamins, bile

acids, fatty acids and eicosanoids with a prominent role in biosynthetic pathways (Liu, 1991; Guengerich, 1999). Some isoforms of CYPs which are involved in different metabolic functions of the body are listed in Table 7.1.

Table 7.1. Human cytochrome P450s involved in metabolic functions.

Main function	Human cytochrome P450
Metabolism and biotransformation of xenobiotics and endobiotics	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP3A7
Fatty acids synthesis	CYP2C8, CYP2C9, CYP2J2, CYP4, CYP5, CYP8A
Steroid synthesis	CYP2G1, CYP7, CYP8B1, CYP11, CYP17, CYP19, CYP21, CYP27A1, CYP46, CYP51
Vitamin D3	CYP24, CYP27B1
Retinoid metabolism	CYP26

7.2.1. Metabolism of xenobiotics by cytochrome P450

CYP 1-3 are the main gene families that are transcriptionnally activated in the oxidative, peroxidative and reductive metabolism and biotransformation of hydrophobic xenobiotics and endobiotics (Nelson *et al.*, 1993). Also involved in the bioavailability and detoxification process are phase II enzymes such as glutathione *S*-transferase, epoxide hydrolase, UDP-glucuronosyltransferase, cysteine conjugate β-lyase, γ-glutamyl transpetidase and methylases (Guengerich and Shimada, 1991).

Several compounds can act as substrates, inhibitors, inducers or activators of CYP enzymes with different biological consequences (Morgan, 2001). Both induction and activation leads to increased catalytic activity of the enzyme but via different mechanisms. The major mechanism of activation is allosteric, whereby the binding of the compound to the enzyme results in a conformational change at the active site of the enzyme. However, induction often involves the hormones and cytokines of the intracellular signalling pathway and often takes a longer period of time to manifest (Hollenberg, 2002). The increased catalytic activity of a CYP enzyme often results in

increased drug clearance, reduced efficacy and side effects (Pelkonen, 2002). However, inhibition of CYP may result in more dramatic consequences to the patient from harmful drug-drug interactions (Pelkonen, 2002). The most common mode of inhibition of CYP enzyme is via a competitive mechanism, with a mechanism-based (suicide) process being less often encountered (Pelkonen, 2002). The biological and clinical consequences of activation, induction or inhibition of CYP enzymes depend on the types of drugs involved, and their therapeutic indices. Some drugs are administered in their active form and are biotransformed by phase I drug metabolising enzymes mainly in the liver to often toxic forms. Inhibition of CYP enzymes responsible for the metabolism of these drugs may result in the accumulation of toxic compounds and have detrimental effects on health. However, some drugs known as prodrugs are metabolised into active forms by CYPs and inhibition of the enzyme is also likely to result in reduced efficacy of the prodrug.

Of the known CYPs found in the liver, CYP3A gene products are the most abundant (Shimada *et al.*, 1994). The CYP3A genes encode for CYP3A4, CYP3A5, CYP3A7 and CYP3A43 with CYP3A4 being the predominant form in adult human liver (Dai *et al.*, 2001). Hepatic CYP3A4 is important in the metabolism of substrates including endogenous steroid substrates, environmental chemicals, carcinogens, phytochemicals and over 50% of therapeutic drugs (Tang and Stearns, 2001). In this study, CYP3A4 has been used as an example to illustrate the regulation of CYPs and its relevance to drug-herb interactions.

7.2.2. Metabolism of arachidonic acid by cytochrome P450

As well as their function in the detoxification of endobiotics and xenobiotics, CYPs are also involved in the metabolism of several biologically significant compounds, including arachidonic acid. In response to stimuli such as cytokines and oxidative stress, arachidonic acid (5,8,11,14-eicosatetraenoic acid) is released from cell membrane glycerophospholipids by hormonally sensitive phospholipases (Chen *et al.*, 2001a; Zeldin, 2001). Arachidonic

acid can be oxidatively metabolised by three enzyme systems, namely, cyclooxygenase (COX), lipoxygenase (LOX) and CYP epoxygenases to a myriad of biologically active metabolites (Capdevila and Falck, 2002). The mode of action of a group of drugs, known as non-steroidal anti-inflammatory drugs (NSAIDs), is inhibition of the action of COX (Hobbs, 1998). Compared to metabolites of COX and LOX, those of CYP epoxygenases are less studied. The CYP pathway was first described in the early 1980's (Capdevila *et al.*, 1981). In general, isoforms of CYP can function as monoxygenases by utilizing NADPH and oxygen in a 1:1 stoichiometry in the presence of one or more electron-transfer proteins (Capdevila *et al.*, 1981; Fulco, 1991). The main products formed from CYP metabolism of arachidonic acid are cis-epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DHET) and hydroxyepoxyeicosanoid (HETE) (illustrated in Fig. 7.1).

7.2.2.1. Main biological functions of EETs

EETs have been reported to be involved in homeostasis in the liver (Zeldin *et al.*, 1996). In addition to the liver, EETs have been detected in several extrahepatic tissues such as the brain, heart, lung and kidney where they elicit physiological responses. For example, EETs have been reported to dilate cerebral arteries mediated through actions on large-conductance Ca^{2+} activated K^+ channels (Alkayed *et al.*, 1996; Harder *et al.*, 1998; Medhora *et al.*, 2001). The EETs regulate renal vasodilation, extracellular fluid, blood pressure and vascular homeostasis (McGiff *et al.*, 1996; Pomposiello *et al.*, 2003). A limited number of studies have shown the anti-inflammatory properties of 11,12-, 8,9- and 5,6-EET in human endothelial cells and the inhibition of the cell adhesion molecule-1 (VCAM-1) (Node *et al.*, 1999). 8,9- and 14,15-EET has also been reported to inhibit COX *in vitro* (Fitzpatrick *et al.*, 1986). The anti-inflammatory properties of 11,12-EET are further evidenced by the *in vitro* inhibition of nuclear factor kappaB (NF-κB) (Node *et al.*, 1999) and the *in vivo* inhibition of PGE_2 (Kozak *et al.*, 2003).

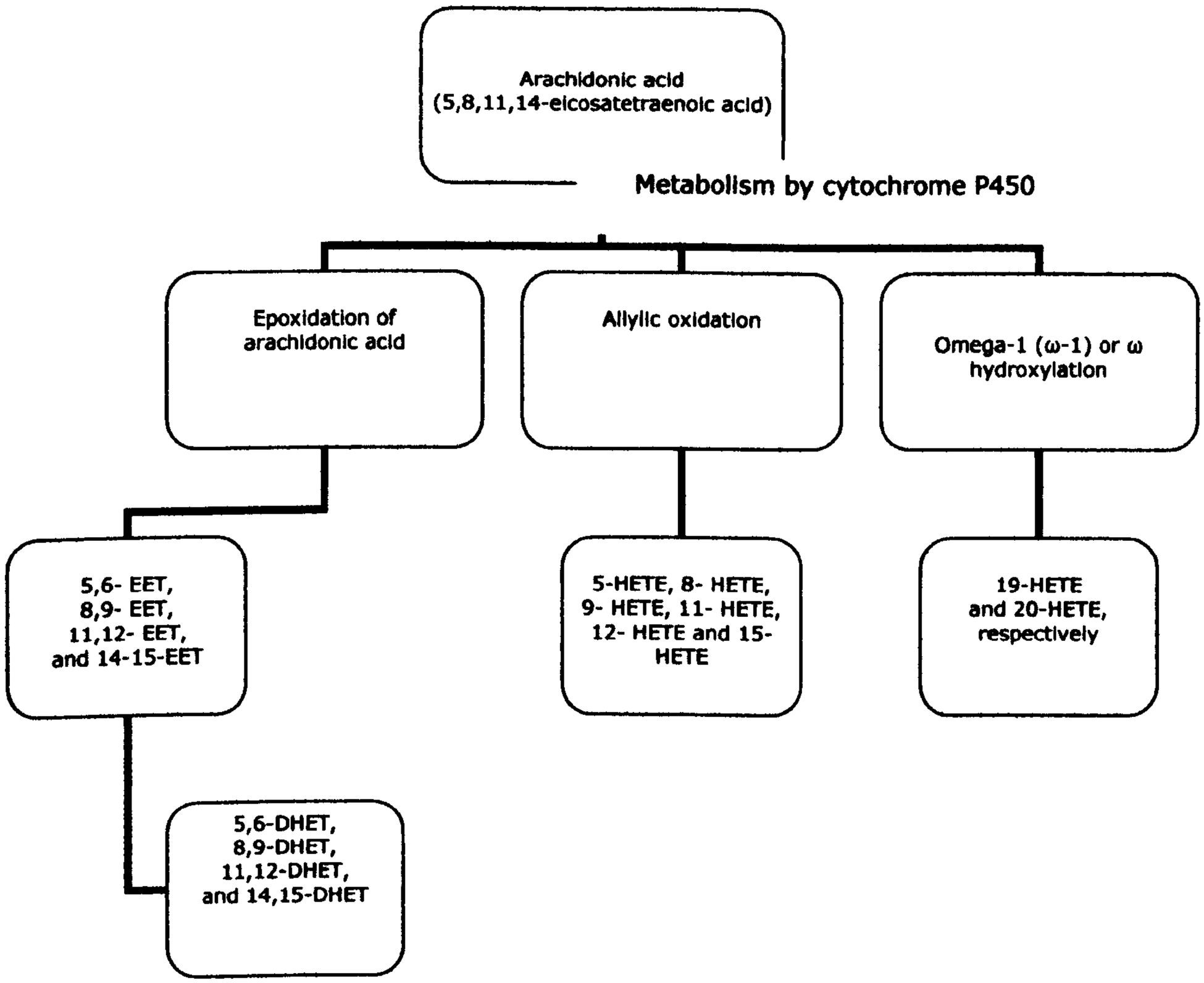


Fig. 7.1. Schematic diagram of arachidonic acid metabolism by cytochrome P450 epoxygenases and hydrolases.
Isoforms of CYP utilize NADPH and oxygen in a 1:1 stoichiometry in three distinct reactions (Capdevila *et al.*, 1988). (1) Epoxidation of arachidonic acid to form four regioisomeric cis-epoxyeicosatrienoic acids (EETs), namely, 5,6-, 8,9-, 11,12-, and 14,15-EET. The EETs are further catalysed by epoxide hydrolases to form vicinal-diols 5,6-, 8,9-, 11,12-, and 14,15-dihydroxyeicosatrienoic acids (DHET). Also, spontaneous non-enzymatic hydration of EETs may result in the production of DHETs, especially for 5,6-EET. (2) Allylic oxidation of a penta-1,4-diene subunit to form 5-hydroxyepoxyeicosanoid (HETE), 8-, 9-, 11-, 12-, and 15-HETE. (3) ω/ω-1 hydroxylation to form 19-HETE and 20-HETE. The metabolites formed and their ratio depends on the profile of the CYP isoforms involved in the reactions. Imbalance between intracellular EETs, DHETEs and HETEs can influence their biological response and result in certain pathological conditions (Kroetz and Zeldin, 2002). The chemical structures for the EETs are illustrated in Fig. 7.8.

Also, 11,12-, 14,15- and 8,9-EET have demonstrated antipyretic effects *in vivo* (Kozak *et al.*, 2000). A by-product of CYP reactions is the formation of superoxide anions, hydrogen peroxide and hydroxyl radicals, as an outcome of NADPH consumption by microsomal monooxygenases (Fleming, 2001). Isoeicosanoids may result from free radical-catalyzed oxidation of arachidonic acid and the clinical relevance of these compounds is currently unclear (Reilly *et al.*, 1998). However, there is sufficient evidence to indicate that the formation of free radicals can be detrimental to health. This process can lead to the activation of NF-κB and the expression of VCAM-1 and is implicated in several diseases including Alzheimer's and Parkinson diseases (Node *et al.*, 1999; Bayol-Denizot, 2000).

7.3. Cytochrome P450 3A4 Inhibition Studies

The standard operating procedure (SOP) for an automated *in vitro* assay for the determination of the concentration of a compound producing 50% inhibition (IC_{50}) of CYP3A4 catalysed 6β-hydroxylations of testosterone (AstraZeneca, Charnwood) was employed. However, the IC_{50} was determined for only ketoconazole, a known inhibitor of the testosterone 6β-hydroxylation, which was used as the positive control. The inhibitory potential of all the other test solutions were tested at single concentrations to enable the screening of 31 herbal extracts and compounds. The incubations were performed using a robotic sample processor (RSP) and they were analysed using a liquid chromatographic-mass spectrometric (LC-MS) method. The analyses were conducted under the supervision of Muir Russell who developed the method for the Development Drug Metabolism & Bioanalysis Section of AstraZeneca R&D, Charnwood.

7.3.1. Samples

Dried hot water extracts of 16 Chinese herbs, two methanolic herbal extracts, water extracts of rhino horn and four TCM prescriptions as well as

four herbal fractions and four compounds were tested (Table 7.2). The herbal extracts and fractions (except SB4v) were prepared as in Section 4.1 and re-dissolved in water to a concentration of 1000 µg/ml (final incubation concentration of 100 µg/ml); fraction SB4v was re-dissolved in 2.5% aqueous acetone. The phytochemicals, baicalin, baicalein, scutellarein and chrysin were also prepared in 2.5% aqueous acetone to an approximate concentration of 200 µg/ml (final incubation concentration of 20 µg/ml).

Table 7.2. Test samples analysed using the CYP3A4 assays

Plant species and Pin yin name	Code	Reference
Lonicera japonica Thunb. (jin yin hua)	JYH	7.6(A)
Glycyrrhiza uralensis Fisch. (gan cao)	GC	7.6(A)
Paeonia suffruticosa Andr. (mu dan pi)	MDP	7.6(A)
Platycodon grandiflorum A.DC. (jie geng)	JG	7.6(A)
Rehmannia glutinosa Steud. (sheng di huang)	SDH	7.6(A)
Acori Graminei Rhizoma (Chang pu)	CP	7.6(A)
Scrophularia ningpoensis Hemsl. (xuan shen)	XS	7.6(A)
Paeonia lactiflora Pall. (chi shao yao)	CSY	7.6(A)
Isatidis Radix (ban lan gen)	BLG	7.6(A)
Coptis Rhizoma (huang lian)	HL	7.3
Anemarrhena asphodeloides Bge. (zhi mu)	ZM	7.3
Phellodendron amurense Rupr. (Huang bai)	HB	7.3
Andrographis paniculata Nees (chuan xin lian)	CXL	7.3
Gardenia jasminoides Ellis (zhi zi)	ZZ	7.3
Scutellaria baicalensis Georgi (huang qin) water extract	SB	7.3
S. baicalensis methanolic extract	SBM	7.4
Fraction 14 from column chromatography of SBM	SB14	7.4
Fraction from column and thin layer chromatography of SBM	SB4v	7.4
Baicalin, 5,6-dihydroxy-7-glucuronide	-	7.4
Chrysin, 5,7-dihydroxyflavone	-	7.4
Baicalein, 5,6,7-trihydroxyflavone	-	7.4
Scutellarein, 5,6,7,4'-tetrahydroxyflavone	-	7.4
Salvia miltiorrhiza crude methanolic extract	SM	7.6(B)
Fraction 7 from column chromatography of SM	SM7	7.6(B)
Fraction 8 from column chromatography of SM	SM8	7.6(B)

Table 7.2. cont'd.

TCM formulation	Code	Reference
Rhino horn (Xi jiao) (RH)	RH	7.5
Qing ying tang	QYT	7.5
Qing ying tang plus RH	QYTRH	7.5
Sheng xi dan	SXD	7.5
Sheng xi dan plus RH	SXDRH	7.5

7.3.2. Preparation of solutions

Phosphate buffer (0.1M; pH 7.4)

Potassium dihydrogen orthophosphate (13.6 g; anhydrous; Sigma, UK) was dissolved in de-ionised water (1 l) to give a stock solution of 0.1 M phosphate. Disodium hydrogen orthophosphate (17.8 g; dihydrate; Fisher Scientific, UK) was dissolved in de-ionised water (1 l) to produce a stock solution of 0.1 M phosphate. A combined phosphate buffer (0.1 M; pH 7.4) was prepared by titrating potassium dihydrogen orthophosphate (0.1 M) against disodium hydrogén orthophosphate (0.1 M) to adjust the pH to 7.4 (using a pH probe).

NADPH regenerating system

In order to maintain a continuous source of β-nicotinamide adenine dinucleotide phosphate (NADPH) necessary for the CYP450 reactions, NADPH regenerating solution was prepared. NADPH (123.88 mg; Sigma, UK) was dissolved in the above phosphate buffer (14.93 ml) to make a 0.1 M, pH 7.4 solution; glucose-6-phosphate (413.65 mg; Sigma, UK) was dissolved in the phosphate buffer (14.67 ml) to make a 0.1M, pH 7.4 solution; magnesium chloride (132.37 mg; Sigma, UK) was dissolved in the phosphate buffer to make a 10 mg/ml solution; glucose 6-phosphate dehydrogenase (250 units; Sigma, UK) was dissolved in the phosphate buffer (1.25 ml) to make a 0.1 M solution. The solutions were mixed in a

ratio of 10:10:10:1 of NADPH, glucose-6-phosphate, magnesium chloride and glucose 6-phosphate dehydrogenase.

Testosterone (20 μM) and ketoconazole (0.1 μM to 100 μM)

Testosterone (29 mg; Sigma, UK) was dissolved in methanol (1 ml; Fisher Scientific, UK) to give a stock solution of 100 mM. Testosterone, 20 μM was prepared by diluting the stock solution (100 mM; 20 μl in 100 ml methanol).

Ketoconazole (0.531 mg; Salford Ultrafine Chemicals, UK) was dissolved in 10 ml dimethyl sulphoxide (DMSO, Fisher Scientific, UK) to give a stock solution of 100 μM. Serial dilutions were made to produce 0.1, 0.3, 1, 3 and 10 μM of ketoconazole.

The purity of the ketoconazole (100 μM) and testosterone (20 μM) solutions were verified by measuring their absorbance using a Spectramax UV spectrophotometer (Molecular Devices Ltd, Sunnyvale, USA). Testosterone was used as the substrate and ketoconazole was the control drug in this assay.

6β-Hydroxytestosterone quality control stocks (10 and 100 μM)

6β-Hydroxytestosterone (1.52 mg; Sigma, UK) was dissolved in methanol (10 ml) to produce a stock solution of 500 μM. Serial dilutions of this stock were performed to give 10 and 100 μM solutions.

Human liver microsomes

Immediately before use, 4 mg/ml of human liver microsomes in phosphate buffer (0.1M; pH 7.4) was prepared. Testosterone (80 μl; 100 mM) was added to human liver microsomes (7.92 ml; 4 mg/ml) to produce a 100 nM solution. The solution was kept on ice prior to use.

7.3.3. Incubations using Tecan Genesis robotic sampling processor

A Tecan Genesis 150 RSP (Tecan UK Ltd, Reading, UK) was used to incubate the test solutions in 96-well plates using an automated set-up and timed procedures. The following sequence was performed, in duplicate, by the RSP. Ketoconazole (0.1, 0.3, 1, 3 and 10 µM), herbal extracts and fractions (1000 µg/ml in water), compounds (200 µg/ml in 2.5% acetone) and negative controls (water or 2.5% acetone) were placed in 96-well plates.

Phosphate buffer (0.1 M; 245 µl) was added to human liver microsomes containing testosterone (100 nM; 50 µl). A test solution (50 µl) was then added. The plates were pre-incubated for 2 minutes. NADPH regenerating mixture (155 µl) was added to each tube (to give a final volume of 0.5 ml) and incubated at 37 $\pm$ 2 ^{o}C for 20 minutes. Phosphoric acid (0.15 M; 0.1 ml) was added to terminate the reactions.

Instrumental quality control samples were performed (for each 96-well plate) by preparing tubes containing the human liver microsomes (50 µl) (with no added testosterone) and phosphate buffer (0.1 M; 245 µl). After the 2 minutes pre-incubation, NADPH mixture (155 µl) was added to each tube (to a final volume of 0.45 ml) and incubated as described above. On the termination of the reactions, by the addition of phosphoric acid, 6β-hydroxytestosterone (50 µl; 10 and 100 µM) was used to generate duplicate quality samples at two concentrations (1 µM and 10 µM). All the incubation tubes were centrifuged at 3000 rpm for 10 minutes at ambient temperature. Aliquots of each sample were placed in HPLC vials using the RSP.

The incubation procedure described above resulted in a 10 times dilution of the test solutions. Therefore, the herbal extracts and compounds were tested at final concentrations of 100 µg/ml and 20 µg/ml, respectively. Baicalin (45 µM), baicalein (74 µM), scutellarein (69 µM) and chrysin (79 µM) were tested. The final concentrations for ketoconazole were 0.01, 0.03, 0.1, 0.3 and 1 µM.

7.3.4. LC-MS method

Liquid chromatographic-mass spectrometric (LC-MS) analysis was conducted using a 1100 HPLC system (Agilent, Berks., UK) interfaced with mass spectrometer. The chromatographic determination of 6β-hydroxytestosterone was performed on a 150 mm x 4.6 mm i.d., 5 µm Luna Phenyl Hexyl column (Phenomenex, Cheshire, UK) with a sentry 20 mm x 2.9 mm Symmetry C_8 guard cartridge (Waters, Mass., USA) at 25 ^{o}C. A flow rate of 1.5 ml/min was used with mobile phase gradient programmed between ammonium acetate buffer (A) and acetonitrile (B). The gradient programme (A:B) was 81:19 (t = 0); 73:27 (t = 3 minutes); 10:90 (t = 10 min) and then back to the original mobile phase ratio of 81:19 at t = 11 minutes till t = 11.5 minutes. The injection volume was 50 µl. The MS detection was made in the positive ion mode, with TurboIonspray, 500^{o}C, flow split 1:5. The nebuliser gas was set at 8 (arbitrary scale), auxiliary gas at 6 L/min, and the curtain gas was set at 10 L/min. The ionspray voltage was 4800 mV, orifice voltage (declustering potential) was 31 mV, ring voltage (focussing potential) was 180 mV and the Q0 voltage (entrance potential) was -5 mV. The ion was monitored at m/z = 305.6 with a scan time of 500 ms. Data were acquired for 10 minutes per sample. The approximate retention time for 6β-hydroxytestosterone was 8.5 minutes.

7.3.5. Data analysis

The peak height of 6β-hydroxytestosterone in each sample was determined using the proprietary Analyst 1.1 software (PE Sciex, Ontario, Canada). The mean response of the blank sample (with no sample) was determined and all peak heights were expressed as a percentage of this value. The values were expressed as mean ± SD. The Student's 1-sample t-test was used to determine statistical differences between test and positive control groups. The difference was considered statistically significant when $p<0.05$. The enzyme kinetic parameter IC_{50} for ketoconazole was calculated using a non-linear method within WinNonLin (Scientific Consulting Inc., North Carolina, USA).

7.3.6. Results

The instrumental quality control (QC) was evaluated from the parameter P in equation 7.1.

$$P = (10 \times L)/H \qquad \text{eq. 7.1}$$

where L and H are the mean responses from QC solutions containing 1 μM and 10 μM of 6β-hydroxytestosterone, respectively. The acceptance range for P was 0.8 to 1.2 (Standard operating procedure, AstraZeneca). The values of P obtained from three separate experiments were 0.9, 0.9 and 1.1 which were within the acceptance criteria.

Ketoconazole, a known inhibitor of CYP3A4 (McKillop *et al.*, 1999) was used as the positive control in the assays. The IC_{50} values determined for ketoconazole, in the three separate experiments were 0.50, 0.53 and 0.62 μM (a typical calibration curve is presented in Fig 7.2). According to the standard operating procedure for the method used to assess the purity of ketoconazole and testosterone standards, the absorbance range for ketoconazole (100 μM) should be within 0.186 – 0.228 AU, with λ_{max} of 303 nm; the values obtained in this study were an absorbance 0.178 AU and λ_{max} of 306 nm. For testosterone (20 μM) the criteria was absorbance within the range, 0.297 – 0.363 AU, with λ_{max} of 242 nm; actual values obtained in this study were an absorbance of 0.283 AU and λ_{max} of 244 nm.

The results for the effect of herbal extracts and compounds on CYP3A4 using testosterone 6β-hydroxylation as a probe for enzyme activity in human liver microsomes (HLMs) are presented in Figs. 7.3 to 7.6. Out of the herbs investigated as substitutes for bear bile, crude hot water extracts of Coptidis Rhizoma (100 μg/ml) significantly reduced CYP3A4 activity by 37% (p=0.01) compared to that of the uninhibited (water) control in human liver microsomes (Fig 7.3). Water extracts of *Gardenia jasminoides*, *Anemarrhena asphodeloides*, *Andrographitis paniculata*, *Phellodendron amurense* and *Scutellaria baicalensis* showed no significant effect on CYP3A4 activity at 100 μg/ml (Fig 7.3). A methanolic extract of *S.*

baicalensis (SBM; 100 μg/ml) and a fraction obtained from the extract (SB4v; 100 μg/ml) inhibited CYP3A4 activity by 31% and 40% ($p<0.05$), respectively (Fig. 7.4). Also, compounds from *S. baicalensis* were tested at concentrations of 20 μg/ml. Baicalin (5,6-dihydroxy-7-glucuronide; 45 μM), a flavonoid constituent of *S. baicalensis*, showed no inhibitory effect on CYP3A4 (Fig. 7.4). Four other constituents of *S. baicalensis* which were tested in this study showed statistically significant inhibitions of CYP3A4 (Fig. 7.4). Chrysin (5,7-dihydroxyflavone; 79 μM) demonstrated the strongest inhibition of 74% ($p<0.001$), compared to the uninhibited (2.5% acetone) control (Fig. 7.4). Baicalein (5,6,7-trihydroxyflavone; 74 μM) and scutellarein (5,6,7,4′-tetrahydroxyflavone; 69 μM) decreased CYP3A4 activity by 41% ($p<0.05$) and 40% ($p<0.05$), respectively (Fig. 7.4).

Water extracts (100 μg/ml) of rhino horn and TCM prescriptions sheng xi dan and qing ying tang (with and without rhino horn) showed no apparent significant effect on the inhibition of 6β-testosterone hydroxylation due to CYP3A4 activity (Fig. 7.5). Out of the 15 water extracts of herbs studied as part of this investigation into alternatives to rhino horn, only *Rehmannia glutinosa* (100 μg/ml) showed any statistically significant effect (12% reduction; $p<0.01$) on CYP3A4 activity compared to the control (water containing no inhibitor) (Figs. 7.3 & 7.6A).

Salvia miltiorrhiza was also investigated as an alternative to rhino horn. A methanolic extract of *Salvia miltiorrhiza* (SM) showed no inhibitory effect on CYP3A4 activity (7.6B). However, two fractions from SM (SM7 and SM8), which had also shown anti-bacterial (Chapter 4) and inhibitory nuclear factor kappaB (Chapter 5) activities in this study caused a decrease in CYP3A4 activity compared to uninhibited controls (Fig. 7.6(B)). Only SM7 demonstrated a statistically significant inhibitory activity (34% reduction; $p<0.05$) (Fig. 7.6(B)).

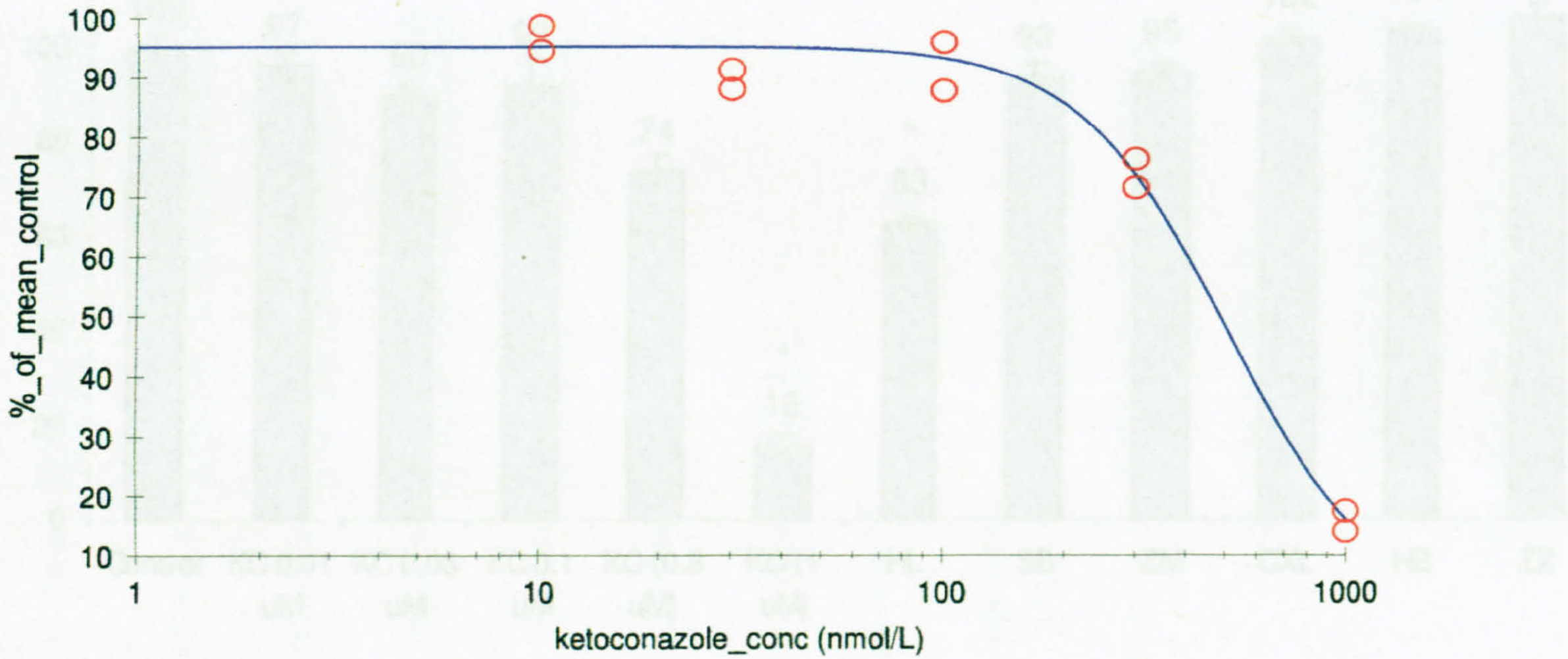


Fig. 7.2. Inhibition of testosterone β-hydroxylation (CYP3A4) by ketoconazole in human liver microsomes.
Different concentrations of ketoconazole were incubated in duplicates with arachidonic acid, testosterone and NADPH in human liver microsomes. LC-MS was used to quantify the amount of 6β-hydroxytestosterone produced. The results were calculated as mean % of uninhibited controls (water).

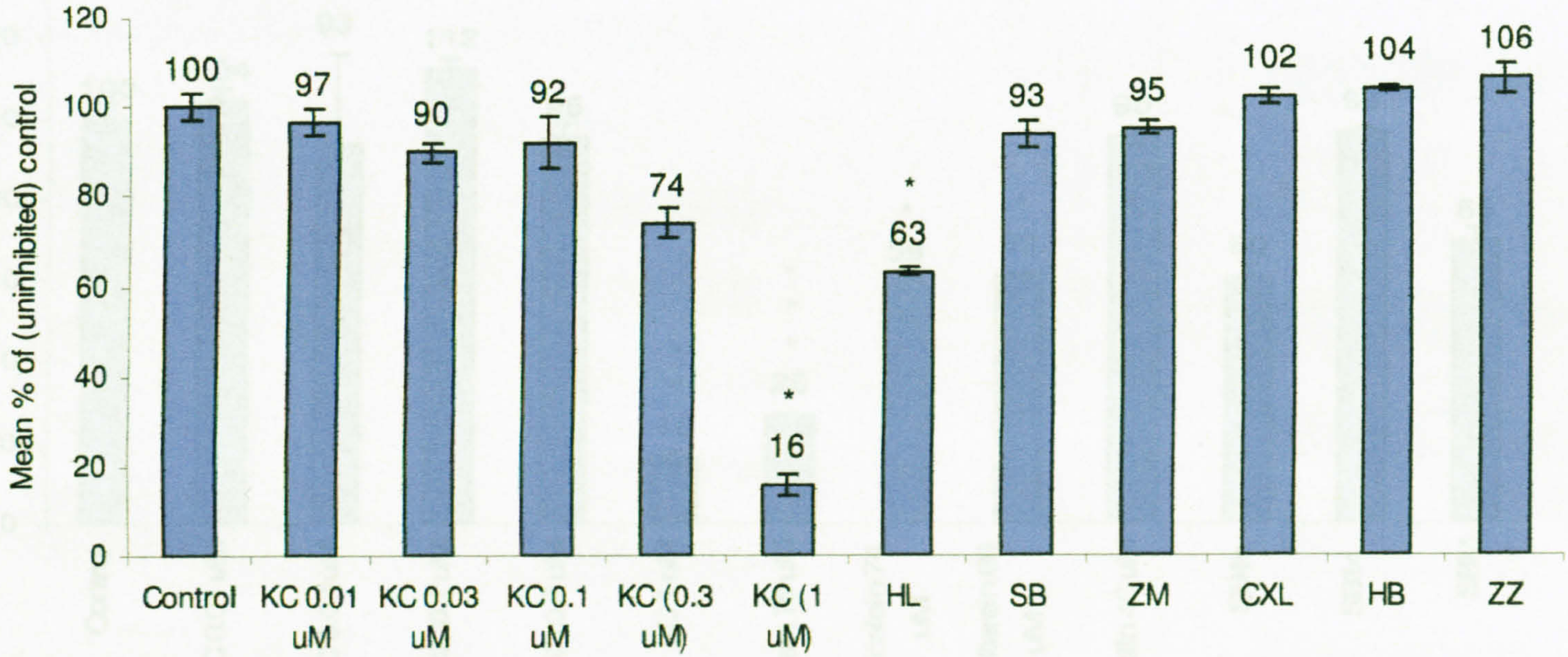


Fig. 7.3. The effects of six TCM herbs and ketoconazole (KC) on CYP3A4 activity.
The assay was conducted using testosterone 6β-hydroxylation as a probe for enzyme activity in human liver microsomes. Results were calculated as mean % of uninhibited controls (water); ketoconazole (KC) was used as positive control. Data represent mean (n = 2) ± SD. *p=0.01 indicates statistically significant differences from groups only treated with water. (The full names of the herbs are described in Table 7.2).

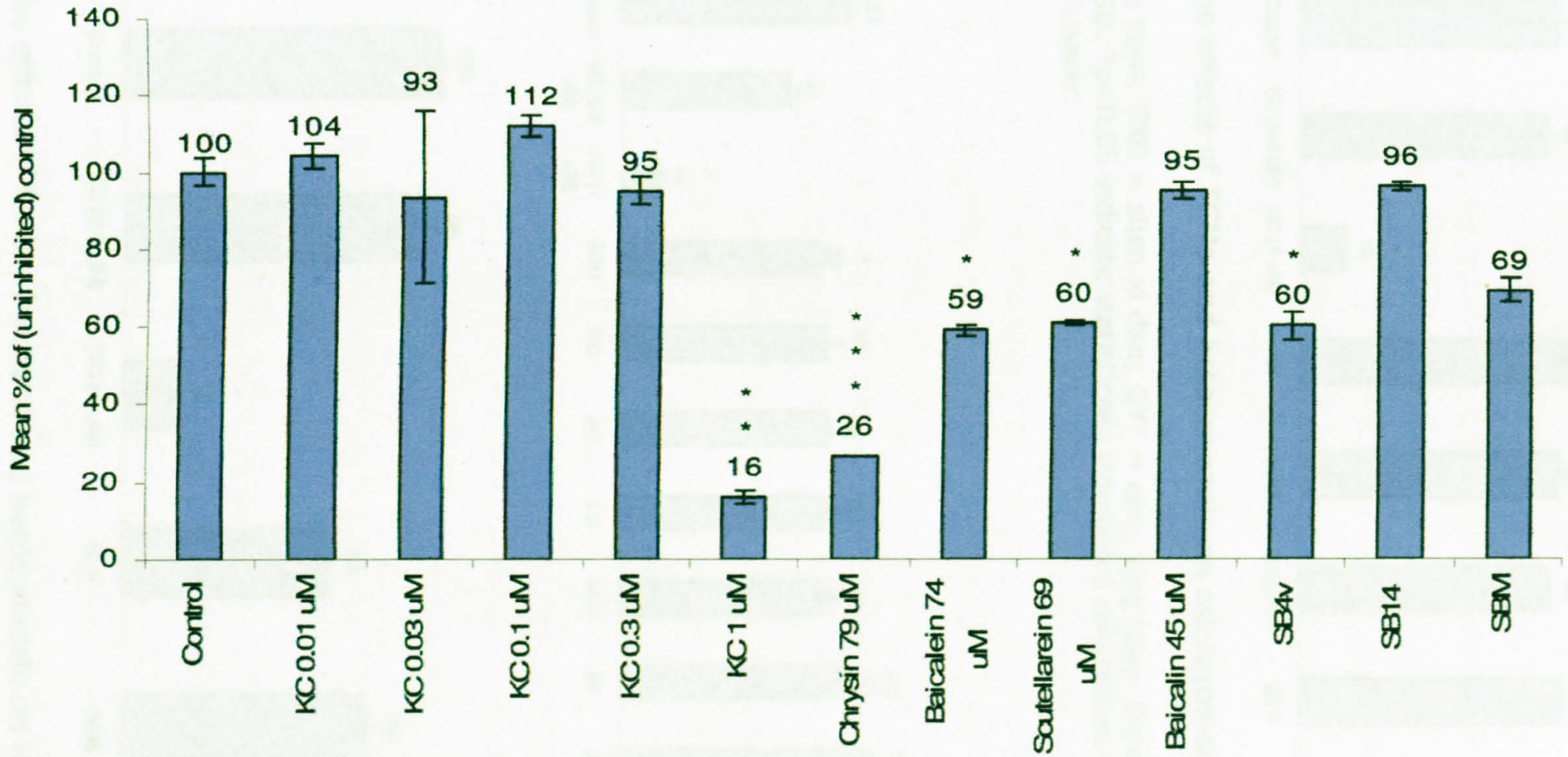


Fig. 7.4. The effects of fractions and flavonoid compounds of *Scutellaria baicalensis* (SB) and ketoconazole on CYP3A4 activity.

The assay was conducted using testosterone 6β-hydroxylation as a probe for enzyme activity in human liver microsomes. Results were calculated as mean % of uninhibited controls (water); ketoconazole (KC) was used as positive control. Data represent mean (n = 2) ± SD. *$p<0.05$, **$p=0.01$, ***$p<0.001$ indicate statistically significant differences from groups only treated with water.

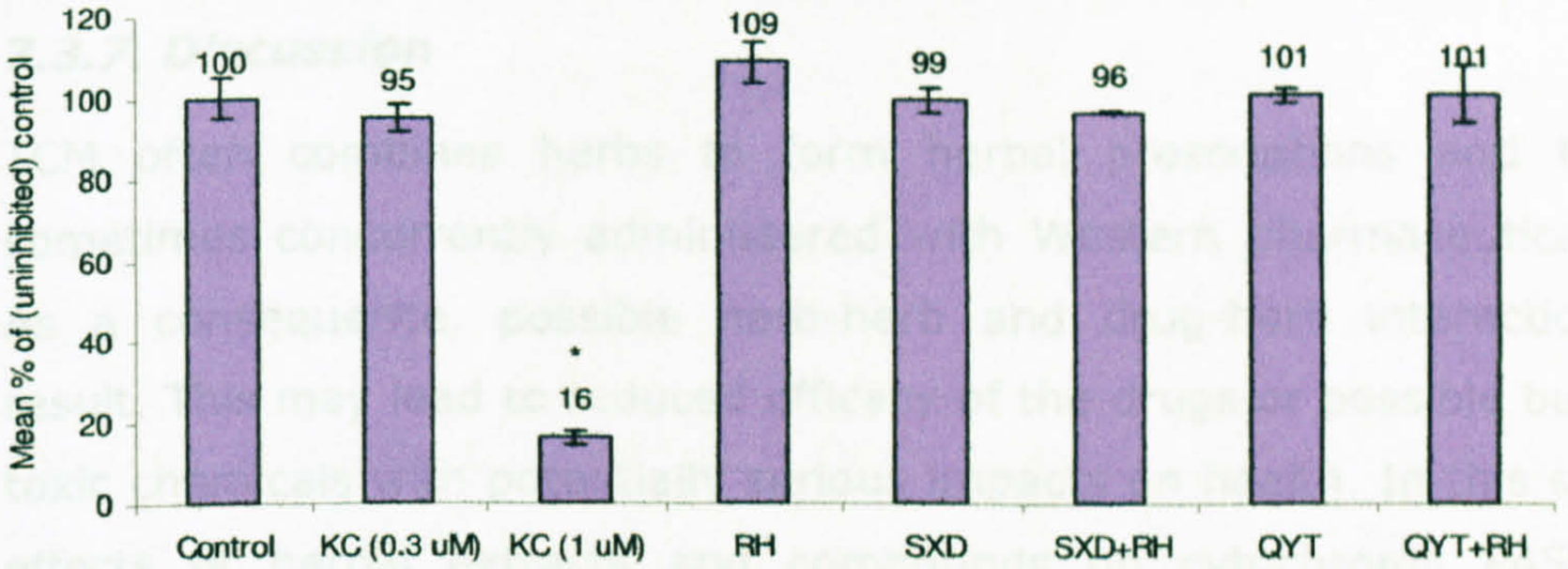


Fig. 7.5 The effects of TCMs and ketoconazole on microsomal CYP3A4 activity.
RH = Rhino horn; SXD = shen xi dan; QYT = qing ying tang. Data represent mean (n = 2) ± SD. *p<0.05 indicate statistically significant differences from groups only treated with water.

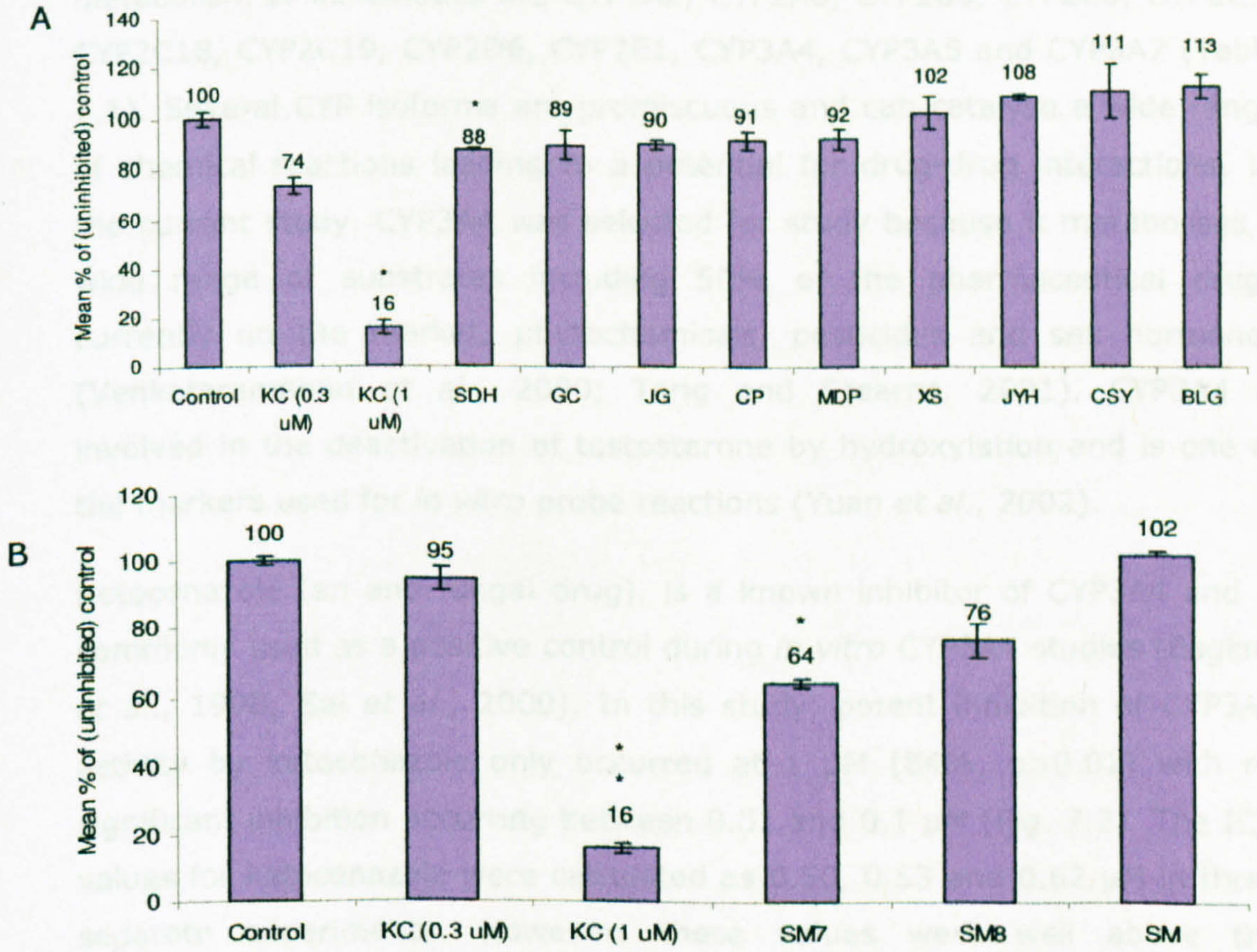


Fig. 7.6 The effects of herbs, fractions and ketoconazole on microsomal CYP3A4 activity.
(A) Nine TCM herbs (full names are described in Table 7.2). (B) Fractions of methanol extract of *Salvia miltiorrhiza* (SM). The data represent mean (n = 2) ± SD. *p<0.05, **p=0.01 indicate statistically significant differences from groups only treated with vehicle solution.

7.3.7. Discussion

TCM often combines herbs to form herbal prescriptions and they are sometimes concurrently administered with Western pharmaceutical drugs. As a consequence, possible herb-herb and drug-herb interactions may result. This may lead to reduced efficacy of the drugs or possible build up of toxic chemicals with potentially serious impacts on health. In this study the effects of herbal extracts and compounds on cytochrome P450s were investigated.

CYPs are the primary drug metabolising enzyme systems involved in the phase I biotransformation of both endogenous and exogenous compounds (Nelson *et al.*, 2004). Some isoforms of CYPs with clinical relevance to the metabolism of xenobiotics are CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7 (Table 7.1). Several CYP isoforms are promiscuous and can catalyse a wide range of chemical reactions leading to a potential for drug-drug interactions. In the current study, CYP3A4 was selected for study because it metabolises a wide range of substrates including 50% of the pharmaceutical drugs currently on the market, phytochemicals, pesticides and sex hormones (Venkataramanan *et al.*, 2000; Tang and Stearns, 2001). CYP3A4 is involved in the deactivation of testosterone by hydroxylation and is one of the markers used for *in vitro* probe reactions (Yuan *et al.*, 2002).

Ketoconazole (an anti-fungal drug), is a known inhibitor of CYP3A4 and is commonly used as a positive control during *in vitro* CYP3A4 studies (Eagling *et al.*, 1998; Sai *et al.*, 2000). In this study, potent inhibition of CYP3A4 activity by ketoconazole only occurred at 1 μM (84%, $p>0.01$) with no significant inhibition occurring between 0.01 and 0.1 μM (Fig. 7.2). The IC_{50} values for ketoconazole were calculated as 0.50, 0.53 and 0.62 μM in three separate experiments. However, these values were well above the acceptance range of 0.08 to 0.32 μM specified by the method used (SOP, AstraZeneca) and published data of 0.1 μM (McKillop *et al.*, 1999) and 0.04 μM (Eagling *et al.*, 1998; Sai *et al.*, 2000). The UV absorbance values obtained for both testosterone and ketoconazole were just outside the

accepted criteria range for the method. This may be an indication of impurities in the standards which could have affected the results. In addition a 40-fold variation of the expression of CYP3A4 in human livers and a 10-fold variation in the metabolism of CYP3A4 substrates have been observed (Dai *et al.*, 2001).

Only two (Coptidis Rhizoma, HL & *Rehmannnnia glutinosa*, SDH) out of the 20 crude TCM water extracts, in addition to fractions from *Salvia miltiorrhiza* and fractions and compounds from *Scutellaria baicalensis* showed statistically significant CYP3A4 inhibition. To date there has been no reports in the English literature on the effect of Coptidis Rhizoma and *Rehmannia glutinosa* on CYP3A4. In a recent report, an active diterpene quinone isolated from *S. miltiorrhiza*, tanshinone IIA, has been shown to decrease and increase protein levels of CYP3A and CYP1A1, respectively, in mice (Ueng *et al.*, 2003; Ueng *et al.*, 2004). *Gardenia jasminoides* was one of the herbs which did not have a significant effect on CYP3A4 activity. However, a water extract of *G. jasminoides* and a constituent of the herb, geniposide, have been reported to decrease rat hepatic CYP3A, although specific isoforms were not determined (Kang *et al.*, 1997). Also, although a water extract of *Glycyrrhiza uralensis* showed no significant inhibitory effect on CYP3A4 in this study (7.6(A)), an ethanolic tincture of *G. uralensis* has been reported to inhibit CYP3A4 with an IC_{50} value of 1.83% of the full tincture strength (Budzinski *et al.*, 2000). Also, a compound isolated from *G. uralensis*, 18 alpha-glycyrrhizic acid (at concentrations of 12.5 μg/g and 50 μg/g, *in vivo*), was reported to have inhibitory activities of on CYP3A, CYP2E1 and CYP1A1 in mice (Yang *et al.*, 2001).

One of the commonly used herbs in TCM is *Scutellaria baicalensis* Georgi and constituents of *S. baicalensis*, namely baicalein (5,6,7-trihydroxyflavone) and 5,7,2',6'-tetrahydroxyflavone have been reported to be inhibit CYP3A4 activity with IC_{50} of 17.4 μM and 7.8 μM, respectively (Kim *et al.*, 2002). In the current study, baicalein, chrysin (5,7-dihydroxyflavone) and scutellarein (5,6,7,4′-tetrahydroxyflavone) all demonstrated an inhibitory effect on CYP3A4 as well as an inhibitory nuclear factor kappaB (NF-κB) effect (Fig. 6.5). Baicalin and and water extract of *S.*

baicalensis (SBW) did not demonstrate a significant inhibitory effect on CYP3A4 or NF-κB activities (Fig. 7.4, 6.3 & 6.5). In studies conducted in China, baicalin was reported to selectively induce CYP1A1, CYP2B1 and CYP2C11 in mice (Hou *et al.*, 2000). The modulation of CYP enzymes by flavonoids isolated from *S. baicalensis* in animal models has also been reported. At very high doses, baicalein (5000 μM; 1351 μg/ml) and wogonin (5000 μM; 1420 μg/ml) both decreased mouse hepatic CYP2E1 and CYP3A protein levels and increased mouse lung CYP1A (Ueng *et al.*, 2000). Mouse hepatic CYP1A2 protein levels were increased by baicalein and decreased by wogonin (Ueng *et al.*, 2000). The structure-activity relationships of several naturally occurring flavonoids on CYPs have been reported (Moon *et al.*, 1998; Breinholt *et* al., 2002; Hodek *et al.*, 2002). Another group of phytochemicals identified as modulators of CYPs are furanocoumarins (Guo *et al.*, 2001; Le Goff-Klein *et al.*, 2004).

The high positive control value observed in this study may indicate that moderate inhibition of CYP3A4 may not have been detected with some of the samples at the concentration tested. Herbs that are mechanism-based (suicide) inhibitors of CYP3A4 would not have been detected in the assay conducted in this study since a pre-incubation time of at least 15 minutes would have been required (Guo *et al.*, 2001). In the current study a pre-incubation time of 2 minutes was used.

In order to predict the threat of possible drug-herb interactions several factors may need to be considered. Herbs pose a particular challenge in the study of CYP activity because they contain several compounds that may interact differently or have similar affinity for different isoforms of CYPs. Apart from drugs, several other factors can influence the expression and activity of CYPs. For example diet, smoking, alcohol, disease, hormones, age, sex and polymorphism, can all affect CYP enzymes and consequently drug clearance and toxin activation (Morgan, 2001). Nutritional factors such as cholesterol and the salt content of food, as well as fasting can be contributing factors to CYP activity (Zeldin, 2001). Some cytokines produced during some disease states can also affect the expression of some CYP enzymes (McGiff *et al.*, 1996). The downregulation of CYPs (including

CYP3A4) in the liver during the host response to infection or inflammation has been critically reviewed by Morgan (2001). In general, females possess a greater catalytic activity of CYP3A4 than males (Parkinson *et al.*, 2004). Genetic polymorphisms in CYP3A4 are not clearly defined. However, one study reported that a variant CYP3A4 L293P had been detected in some Asians but not in Caucasians and the variant CYP3A4 F189S was specific to the Caucasian population (Dai *et al.*, 2001). When tested on their metabolism of testosterone and the pesticide chlorpyrifos, CYP3A4 L293P had higher turnover numbers for both substrates than CYP3A4 F189S (Dai *et al.*, 2001). The implication of genetic polymorphism is that certain drug-herb interactions may pose greater threats to certain individuals or ethnic groups than others. In this study, individual water extracts *Scutellaria baicalensis*, Coptidis Rhizoma, *Salvia miltiorrhiza* and *Rehmannia glutinosa* all caused inhibitions of CYP3A4. However, the implications in drug-herb or herb-herb interactions may be different for different people.

7.4. Arachidonic Acid Metabolism to Epoxyeicosatrienoic Acids

The incubation method used for the production of epoxyeicosatrienoic acids (EETs) from arachidonic acid provides a manual version of the automated method used in the CYP3A4 inhibition assay (Section 7.3.3). The extraction and analysis techniques followed those described by Nithipatikom et al. (2001) with slight modifications. The analyses were conducted under the supervision of Dr Victoria Holmes (AstraZeneca, R&D, Charnwood).

7.4.1. Samples

Miconazole (nitrate salt; Sigma), indomethacin (Sigma, UK), ursodeoxycholic acid (UDCA, Sigma, UK), water extracts of *Scutellaria baicalensis*; water extract of herbal prescription X (equal quantities of five

herbs namely Isatidis Radix, *Lonicera japonica*, Coptis Rhizoma, *Gardenia jasminoides and Forsythia suspensa*) were tested.

7.4.2. Preparation of samples and solutions

Phosphate buffer (0.1 M; pH7.4) and NADPH-regenerating mixture were prepared as detailed in Section 7.2.2. Arachidonic acid (30 µl; Biomol, USA) was dissolved in DMSO (970 µl) to provide a stock solution of 100 mM. Standards for 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET were obtained as 50 µg/ml solutions in ethanol (Biomol, USA). The four EETs were mixed to form five calibration standard solutions with concentrations of 1, 5, 10, 30 and 60 ng/ml in acetonitrile.

Duplicate samples of each of the following test solutions were prepared in microtubes on ice: miconazole (1 mM in DMSO; 5 µl), indomethacin (50 mM in DMSO; 5 µl), UDCA (50 mM in DMSO; 5 µl), water extract of *S. baicalensis* (5 µl of 1 mg/ml in H_2O, plus 5 µl DMSO), water extract of prescription X (5 µl of 2 mg/ml in H_2O, plus 5 µL DMSO) and blank samples (10 µl of DMSO).

7.4.3. Incubation method

To each test solution (Section 7.4.2), arachidonic acid (5 µl) was added and kept on ice. In addition, 11 microtubes containing 10 µl DMSO (with no added arachidonic acid) were prepared for the calibration standards. One of these tubes was used as a further blank sample and 10 were used to generate five duplicate standard solutions at the end of the incubation period. Human liver microsomes (4 mg/ml; 25 µl) and phosphate buffer (0.1 M, pH 7.4; 310 µl or 305 µl for water extracts) were also added to each of the mixtures (sample tubes were kept on ice). The solutions were pre-incubated for 2 minutes at 37°C in a gently shaking water bath. NADPH regenerating mixture (155 µl) was then added to each tube (to give a final volume of 0.5 ml) and incubated further at 37°C for 20 minutes. Ethanol (200 µl) was added to terminate the reactions and the solutions vortex

mixed and stored on ice. Calibration standard solutions (5 µl) were added to the appropriate solutions to provide five duplicate standard solutions (from individual stock solutions of 0.1, 0.5, 1, 3 and 6 µg/ml). Tridecanoic acid (5 µl; 0.5µg/ml) was added to each of the incubation solutions.

7.4.4. Extraction

Solid phase extraction C_{18} Bond Elut columns (Anachem, Luton, England) were preconditioned with 4 ml of ethanol and 12 ml of water. The previously incubated microsomes were mixed, applied to the columns and allowed to run dry. The columns were washed with 6 ml water and the washings collected in plastic tubes and the column was run dry. The EETs were eluted with ethyl acetate (4 ml) into screw top plastic tubes. The water washings (initially collected in the plastic tubes) were re-extracted with ethyl acetate (5 ml). The ethyl acetate fractions were combined and dried under nitrogen. The dried extract was re-constituted with acetonitrile (50 µl) and transferred into HPLC vials for LC-MS analysis. To compare recoveries and confirm retention times, separate vials containing 1, 5, 10, 30 and 60 ng/ml of individual EETs (5, 6-, 8,9-, 11,12- and 14,15-EET) standards were also prepared in acetonitrile.

7.4.5. LC-MS method

Liquid chromatographic-mass spectrometric (LC-MS) analysis was conducted using an 1100 LC system interfaced with a 1100 Mass Selector detector (Agilent, Berks., UK). The chromatographic separation of the EETs was performed on a 250 mm x 2.1 mm i.d., 5 µm Kromasil C18 column (Hichron, Reading, UK) with a corresponding guard column (Hichron, Reading, UK) at 25 ^{o}C. A flow rate of 0.200 ml/min was used with isocratic mobile phase composed of water/acetonitrile (2:3) containing 0.05% acetic acid. Data were acquired for 55 minutes per sample. The injection volume was 5 µl with acetonitrile wash. As an instrument blank, acetonitrile was also run. Drying gas flow was 10 litres/min, drying gas temperature was

350^{O}C, nebulizer pressure was 35 psig, capillary voltage was 3500V, and fragmentor voltage was 100 V. The detection was made in the negative mode. For quantitative measurements, the *m/z* = 319.0 (80% dwell time) and 213.0 (20% dwell time) ions were used for EETs.

7.4.6. Data analysis

The peak height of each EET and the internal standard (tridecanoic acid) were determined using ChemStation software. The ratios of peak heights of the EETs to that of the internal standard were determined. Calibration curves were constructed for each EET (5, 6-, 8,9-, 11,12- and 14,15-EET) over the concentration range of 1 to 60 ng/ml. The concentrations of the EETs in the samples were calculated by comparing the ratios of peak heights to the standard curves. The mean response of the blank sample containing arachidonic acid (with no sample) was calculated. The average peak ratios of the samples were expressed as a percentage of that of the blank. The values were expressed as mean $\pm$ SD.

7.4.7. Results

Epoxyeicosatrienoic acids (EETs) formed from the oxidative metabolism of arachidonic acid (AA) by cytochrome P450 (CYP) enzymes present in human liver microsomes (HLMs) in the presence of NADPH were determined. A typical LC-MS chromatogram is shown in Fig. 7.7, in which the EETs in a blank and ursodeoxycholic acid (UDCA) sample are compared. The effect of the test samples on EET production was expressed as the mean % of the blank control (Fig. 7.9). Good linear relationships were obtained for the EETs in the concentration range of 1-60 ng/ml in HLMs: 14,15-EET (y=0.104x + 0.215, r=0.995); 11,12-EETs (y=0.033x + 0.239, r=0.979); 8,9-EETs (y=0.035x+0.276, r=0.994) and 5,6-EETs (y=0.107x+0.754, r=0.998). The recoveries of EETs from HLMs, at 60 ng/ml, were 14,15-EET (71.4%); 11,12-EETs (77.4%); 8,9-EETs (97.1%) and 5,6-EETs (61.0%). The chemical structures of the EETs are given in Fig. 7.8.

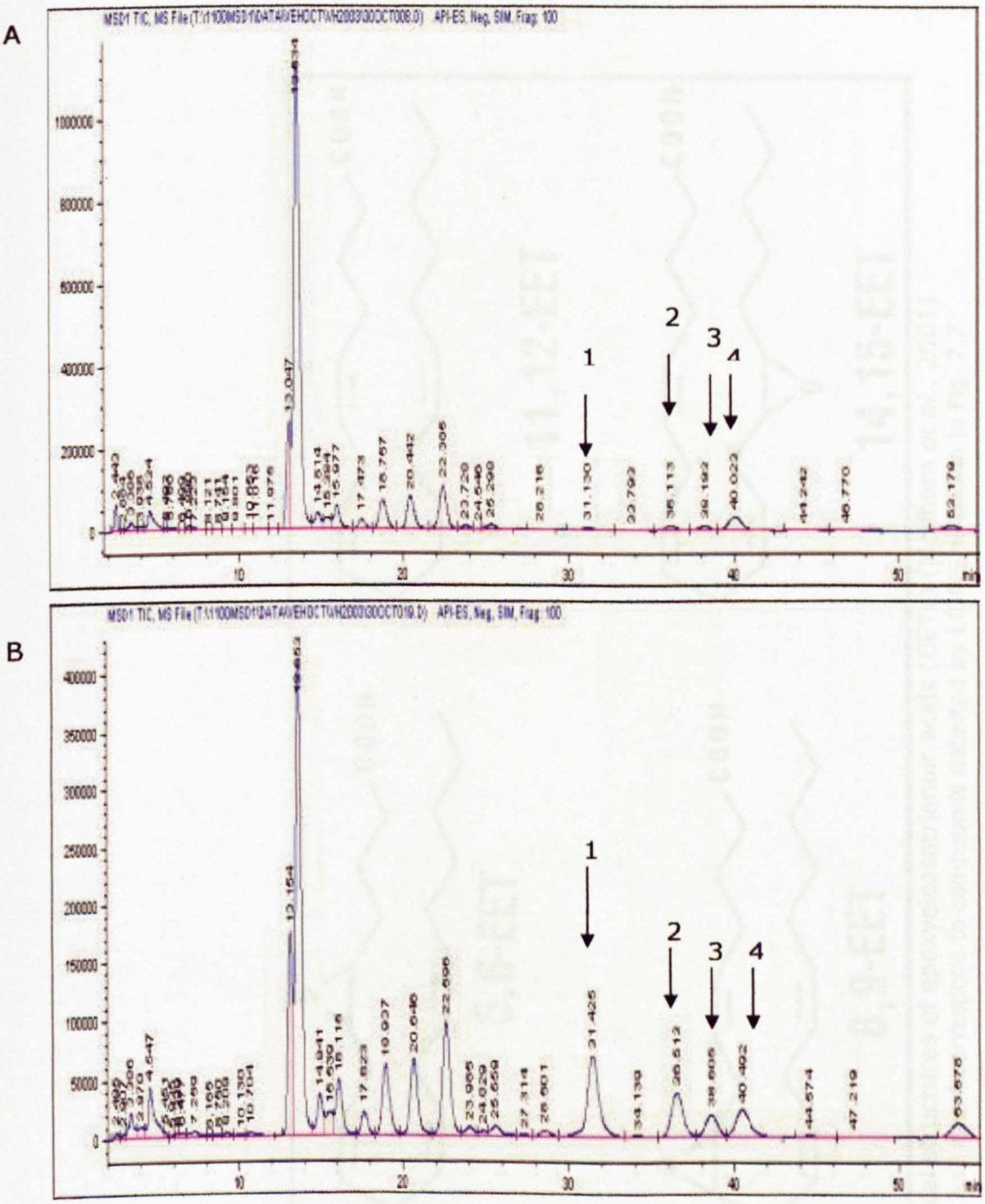


Fig. 7.7. LC-MS chromatogram of epoxyeicosatrienoic acids (EETs).
(A) Blank sample (DMSO with no arachidonic acid) and (B) Ursodeoxycholic acid (UDCA) solution containing arachidonic acid. The samples (in DMSO) were incubated with human liver microsomes in the presence of NADPH as described in Section 7.4.3. (1) 14,15-EET (2) 11,12-EET (3) 8,9-EET and 5,6-EET were quantified using LC-MS in specific ion detection mode m/z = 319.0 (80% dwell time) and 213.0 (20% dwell time).

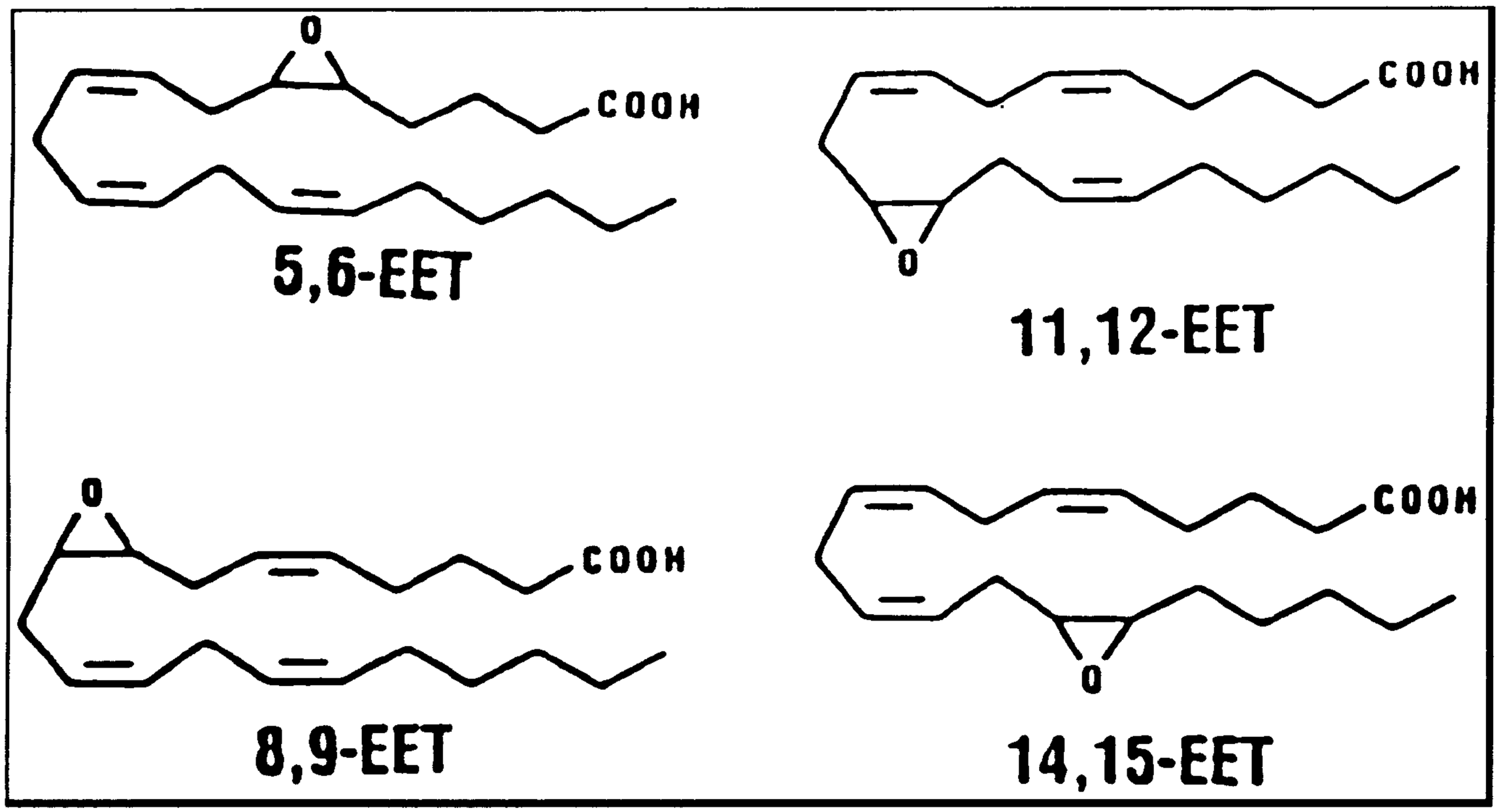


Fig. 7.8. Chemical structures of epoxyeicosatrienoic acids (EETs) (Medhora *et al.*, 2001).
The structures 1, 2, 3 and 4 correspond to compounds detected by LC-MS as shown in Fig. 7.7.

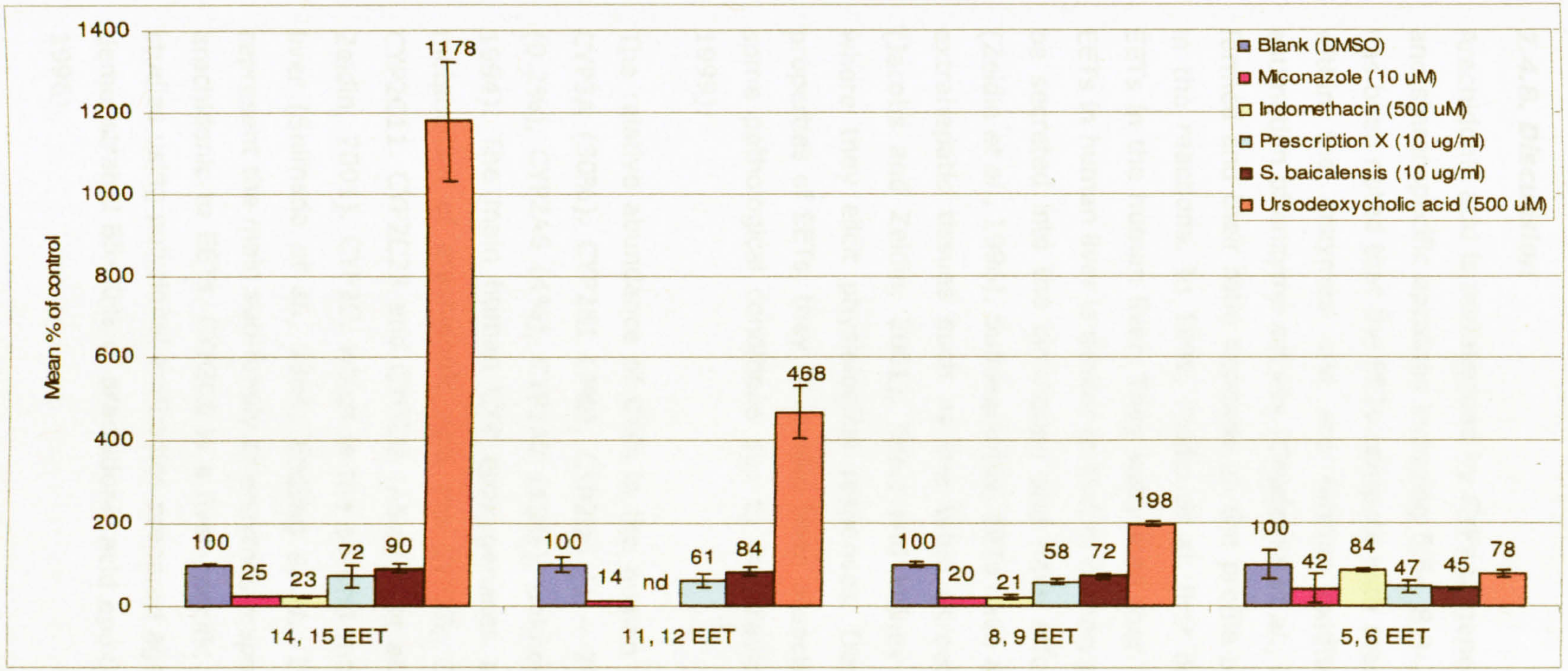


Fig. 7.9. The effect of compounds and herbal extracts on cytochrome P450-catalysed production of epoxyeicosatrienoic acids (EETs).

Pharmaceutical drugs, miconazole, indomethacin and ursodeoxycholic acid as well as herbal extracts of *Scutellaria baicalensis* and prescription X were tested for their effect on EET production from arachidonic acid in human liver microsomes as described in Section 7.4.3. The results were calculated as mean % of uninhibited controls (DMSO). Data represent mean (n = 2) ± SD, except for miconazole where n = 1 for 14,15-EET, 11,12-EET and 8,9-EET. The herbal composition of prescription X is equal quantities of Isatidis Radix, *Lonicera japonica*, Coptis Rhizoma, *Gardenia jasminoides and Forsythia suspensa*.

7.4.8. Discussion

Arachidonic acid is metabolised by CYPs to generate a series of regiospecific and stereospecific epoxides including 5,6-, 8,9-, 11,12-, and 14,15-EETs. It has been noted that the EETs compete with arachidonic acid for active sites within the enzymes and are further oxidised which complicates the estimation of enzyme activity (Capdevila *et al.*, 2000). Also, the metabolites formed and their ratio depends on the profile of the CYP isoforms involved in the reactions. In 1996, Zeldin et al. first documented the presence of EETs in the human liver. They suggested that "since the regiochemistry of EETs in human liver is similar to that in human plasma ...that liver EETs may be secreted into the circulation and have effects in extrahepatic tissues" (Zeldin *et al.*, 1996). Subsequently, EETs have also been detected in several extrahepatic tissues such as the brain (Kroetz and Zeldin, 2002), lung (Jacobs and Zeldin, 2001), heart and kidney (Pomposiello *et al.*, 2003) where they elicit physiological responses. Despite the clinically relevant properties of EETs, they have also been associated with the progression of some pathological conditions due to free radical production (Node *et al.*, 1999).

The relative abundance of CYPs in the human liver has been described as CYP3A (30%), CYP2E1 (7%), CYP2D6 (1 – 2%), CYP2C (18%), CYP2B6 (0.2%), CYP2A6 (4%), CYP1A2 (13%), unknown (28%) (Shimada *et al.*, 1994). The main human CYP epoxygenases sub-families involved in the metabolism of arachidonic acid are CYP2B, CYP2C8, CYP2C9, CYP2C10, CYP2C11, CYP2C23 and CYP2J2 (Alkayed *et al.*, 1996; Node *et al.*, 1999; Zeldin, 2001). CYP2C, which is the second most abundant CYP in human liver (Shimada *et al.*, 1994; Eagling *et al.*, 1998) has been reported to represent the main sub-family of enzymes responsible for the epoxidation of arachidonic to EETs. CYP2C8 is a liver-specific hemoprotein and "inhibition studies using polyclonal antibodies prepared against recombinant CYP2C8 ... demonstrated 85-90% of arachidonic acid epoxide formation" (Zeldin *et al.*, 1996).

In the current study the effects of compounds and herbal extracts on the production of EETs were investigated. Miconazole (an anti-microbial agent) is a known inhibitor of CYP epoxygenase (Alkayed *et al.*, 1996) and was used as a positive control. Miconazole potently reduced the production of 14,15-, 11,12-, 8,9-, and 5,6-EET by 88%, 93%, 90% and 58%, respectively, compared to those by uninhibited (blank) samples (Fig. 7.9).

Indomethacin, a known COX inhibitor, is metabolised by CYPs into inactive forms of *O*-desmethylindomethacin and *N*-deschlorobenzoylindomethacin, and subsequently to *O*-desmethyl-*N*-deschlorobenzoylindomethacin for elimination from the body (Duggan *et al.*, 1972). It has been reported that *O*-demethylation accounts for 40-55% of total indomethacin eliminated in urine and in humans this reaction is catalysed exclusively by CYP2C9 (Nakajima *et al.*, 1998). Interestingly, in the present study indomethacin, completely inhibited the production of 11,12-EET and inhibited 14,15-, and 8,9-EET by 77%, 79%, respectively, compared to those by uninhibited samples (Fig. 7.9). However, indomethacin had only a marginal effect on the production of 5,6-EET (Fig. 7.9). Being a substrate for CYP2C9, the inhibition of the 11,12-, 14,15-, and 8,9-EET by indomethacin may be via competitive inhibition. If this assumption is correct, then the current research has provided further evidence of the significant contribution of CYP2C9 in the metabolism of arachidonic acid to EETs. Water extracts of *Scutellaria baicalensis* and a herbal prescription decreased the production of all the EETs but to different extents (Fig. 7.9). The herbal extracts produced a reduction of 5,6-EETs by more than 50% which was similar to that achieved by the positive control, miconazole (Fig. 7.9). Although methanol extracts of *Scutelaria baicalensis* significantly inhibited testosterone 6β-hydroxylation (CYP3A4) by 31%, in contrast the water extracts had no effect on CYP3A4 (Fig. 7.3) but the water extracts showed some inhibitory effects on the EETs as mentioned above. The results may indicate that some polar compounds from *Scutelaria baicalensis* are substrates and/or inhibitors of CYPs involved in the formation of EETs, most likely in the CYP2C family. Another compound tested in the assay was the bile acid ursodeoxycholic acid (UDCA), an active constituent of bear bile. UDCA greatly increased the production of 14,15-EETs by about 12-fold, 11,12-EET

by 5-fold and 8,9-EETs by 2-fold but inhibited 5,6-EET by 22% (Fig. 7.9). UDCA probably caused the increase in the production of the EETs by stimulation (allosteric) rather than induction (which takes several hours to weeks to manifest). However, further work is required before the results can be fully interpreted. In these preliminary studies, the assay was not designed to determine the specific enzymes being inhibited. However, as discussed above, from published literature it was deduced that the CYP2C subfamily is likely to be predominant in the production of EETS in the adult human liver. CYP2C8 is involved in the metabolism of several drugs including rosiglitazone, an anti-diabetic drug (Park *et al.*, 2004) and taxol, an antitumor drug also known as paclitaxel (Goldstein, 2001). In addition, various CYP3A4 inhibitors have been reported to be mechanism-based inhibitors of CYP2C8 (Polasek *et al.*, 2004). Therefore, drug-drug interactions of the isoform CYP2C8 and the CYP2C subfamily are of great clinical relevance.

7.5. Conclusions

Preliminary results from this study indicate that when extracts of the herbs, *Scutellaria baicalensis* (huang qin), Coptidis Rhizoma (huang lian), *Salvia miltiorrhiza* (dan shen) or *Rehmannia glutinosa* (sheng di huang) are co-administered with drugs metabolised by CYP3A4 with a narrow therapeutic index, possible drug-herb interactions may result. The Kampo prescriptions diao-orengedokuto and orengedokuto, which have also been suggested as herbal alternatives to bear bile (Table 8.2), both contain Coptidis Rhizoma and *Scutellaria baicalensis*. It is possible that herb-herb interactions may occur in these preparations. However, further work is required to investigate the extent of these effects. Extracts of prescription X and *Scutellaria baicalensis* caused a decrease in the level of EETs produced from arachidonic acid. These reactions were likely to be metabolised in part by the CYP2C subfamily which are also involved in the metabolism of some pharmaceutical drugs.

Chapter 8. Conclusions and recommendations for further research work

8.1. Conclusions

The primary aim of this research was to conduct studies to identify herbal alternatives to the use of both bear bile and rhino horn, derived from endangered species, in traditional medicine. As outlined in Section 1.4, eight objectives were used as a basis for this research.

An important aspect of this work was to be able to promote the findings effectively to practitioners and patients of traditional medicine. Therefore, the herb selection was based on an extensive literature review as well as on TCM philosophy and practice through seeking guidance from TCM practitioners and lecturers. Both bear bile and rhino horn are traditionally reputed to possess antipyretic, anti-bacterial and anti-inflammatory properties and over 100 herbs were identified from initial literature searches as reputed to possess these properties. Applying TCM philosophy to compare the functions and the properties of the medicinal herbs to those of the animal products proved effective in narrowing down the number of selected herbs to 24.

The identification of the plant species used to prepare the herbs enabled the study of the correct species and also facilitated literature searches. Seventeen of the 24 herbs were verified as prepared from plant species listed in Chinese Pharmacopoeia and Materia Medicas by comparing their chemical 'fingerprint' profiles to those of reference samples. Some groups of compounds identified from the UV profiles of the 'fingerprints' also gave some indication of the potential pharmacological effects of the herbs.

Concentrations of metal and pesticide residues in selected herbs were measured as a further quality check on TCM herbs being supplied to the UK. It was concluded that the herbs did not contain any of the 125 pesticide residues tested and the concentration of heavy metals such as Hg, Cd, Sn and Cu were below the current maximum permitted concentrations either in the UK or in China. In contrast, analysis of rhino horn showed

concentrations of Hg, Cd, Sn and Cu above the maximum legally permitted concentrations. However, since the rhino horn sample investigated was part of a sample confiscated by the CITES branch at Heathrow airport, the storage conditions could have contributed to the elevated concentrations of metals.

Studies were conducted to evaluate the anti-bacterial activity of the herbs and to investigate a possible mechanism of anti-inflammatory action of the herbs using nuclear factor kappaB (NF-κB) assay. In addition, original studies were conducted to assess and compare the effects of prescriptions with or without rhino horn and rhino horn alone on bacterial growth and NF-κB activity. Rhino horn when tested alone did not demonstrate anti-bacterial or inhibitory NF-κB activity in the assays adopted in this study. However, some TCM herbal prescriptions containing rhino horn and some without the animal product showed anti-bacterial or inhibitory NF-κB activity. It was concluded that some TCM prescriptions documented to contain rhino horn could still be efficacious as anti-bacterial or anti-inflammatory agents in the absence of the animal product. When herbs were tested individually, the crude ethyl acetate extracts of seventeen showed anti-bacterial effects and crude water extracts of nine demonstrated inhibitory NF-κB activity effects.

Although the herbs are often administered as water decoctions in traditional medicine, in an *in vitro* assay the active constituents of the herbs could be masked among the numerous compounds in the extracts, leading to false negatives. This assumption was shown to be correct in this study when fractions and compounds obtained from the crude extract of *Scutellaria baicalensis* demonstrated inhibitory NF-κB activity but the crude extract showed no inhibitory NF-κB activity. It was beyond the scope of this current study to fractionate all the herbs studied. Nevertheless, crude extracts of 19 out of the 24 herbs investigated showed anti-bacterial and/or inhibitory NF-κB effects.

Before proposing herbs as alternatives to animal products an important consideration is the potential risk of adverse effects due to the combination of the herbs (which is a common practice in TCM) or when the herbs are

combined with Western pharmaceutical herbs. An assay developed as part of this study indicated that *Scutellaria baicalensis* could also affect drug metabolising enzymes involved in the metabolism of arachidonic acid, which were postulated to be CYP2C8 and CYP2C9. Also, Results showed that crude water extracts of *Scutellaria baicalensis*, *Salvia miltiorrhiza*, *Rehmannia glutinosa* and Coptidis Rhizoma caused inhibition of cytochrome P450-3A4 (CYP3A4) enzyme activity in human liver microsomes. CYP3A4, CYP2C8 and CY2C9 are major drug metabolising enzymes involved in the detoxification of several pharmaceutical drugs. Therefore, if any of the above mentioned herbs are combined or if co-medicated with pharmaceutical drugs, metabolised by the indicated CYP enzymes with a narrow therapeutic index, it could lead to build up of toxic chemicals in the liver. In this study, two TCM prescriptions (Table 8.2) which have been proposed as possible alternatives to bear bile contains *Scutellaria baicalensis* and Coptidis Rhizoma among other herbs. Therefore, preliminary results indicate that possible adverse herb-herb interactions, due to CYP3A4 activity may require further investigations. It will be erroneous to draw absolute conclusions from these preliminary studies since there are other factors to consider such as the lifestyle of an individual and genetic polymorphism which indicate that certain human races may metabolise drugs differently from others.

Supported by evidence of efficacy as indicated by inhibitory NF-κB activity and/or anti-bacterial activity as measured in this study, by information obtained from the available scientific literature, and by TCM theory, a number of prescriptions and single herbs have been selected as suitable alternatives to the use of bear bile and rhino horn. Most of the suggested herbal 'alternatives' to the animal products were found to already form part of one or more traditional prescriptions containing the animal products. This finding confirmed the practice in TCM of combining remedies with similar functions for their additive and synergistic effects. The individual herbs proposed as alternatives to the animal products (listed in Tables 8.1 and 8.3, for bear bile and rhino horn, respectively) and the original selection (Tables 2.2 and 2.6, for bear bile and rhino horn, respectively) based on TCM philosophy, were mostly in agreement. There were only two exceptions. The herb tian hua fen, Trichosanthis Radix (which was

investigated because it formed part of the prescriptions studied) demonstrated potent NF-κB inhibitory activity in this study and added to the list of herbal alternatives to bear bile. The inhibitory NF-κB activity and anti-bacterial effects of the root *Paeonia veitchii* were not confirmed in this study and the herb was therefore removed from the list of herbal alternatives to rhino horn. The relevant published literature, as well as results from the current study of the herbs are summarised in Appendices IX and X for herbs proposed as alternatives for bear bile and rhino horn, respectively.

8.1.1. Herbs proposed as potential alternatives to bear bile

Eight individual herbs (Table 8.1) are proposed as potential alternatives to bear bile. In addition to the single herbs, two Kampo patent medicines (Table 8.2) consisting of a combination of herbs studied individually in this study are proposed as alternatives to bear bile based on published TCM and other scientific literature (as summarised in Appendix IX). Bear bile is often used in combination with other TCM herbs and it is also sometimes used as a singly. Substitution of bear bile in these preparations may require a combination of herbs based on TCM philosophy. Combinations of the herbs and appropriate doses would be best determined by TCM practitioners based on the patient's symptoms.

The Association of Chinese Medicine and Philosophy and Earthcare Society (Hong Kong) has published a report on 'Herbal alternatives to bear bile in Chinese medicine' (IFAW report, 1994). This report suggested 54 herbs as alternatives to bear bile (Appendix II) based on TCM philosophy. In the present study, herbs are proposed as possible alternatives to the animal products based on both TCM philosophy and biomedical research. Five of the eight herbs listed in Table 8.1 were also proposed in the IFAW report as alternatives to bear bile. The herbs are chuan xin lian, da huang, huang bai, huang qin and, zhi zi.

Table 8.1. Single herbs proposed as potential alternatives to bear bile

Name, plant part and family of herb
1. Chuan xin lian, aerial part of *Andrographis paniculata* Nees (Acanthaceae)
2. Da huang, root and rhizome of *Rheum palmatum* L. (Polygonaceae)
3. Huang bai, cortex of *Phellodendron amurense* Rupr. or *P. chinense* Schneid. (Rutaceae)
4. Huang lian, rhizome of *Coptis chinensis* Franch. (Ranunculaceae)
5. Huang qin, root of *Scutellaria baicalensis* Georgi (Labiatae)
6. Tian hua fen, root of *Trichosanthes kirilowii* Maxim (Cucurbitaceae)
7. Zhi mu, rhizome of *Anemarrhena asphodeloides* Bge. (Anthericaceae)
8. Zhi zi, fruit of *Gardenia jasminoides* Ellis (Rubiaceae)

Table 8.2. Prescriptions proposed as potential alternatives to bear bile based on the available published literature

TCM names of herbs included in prescription	Name of prescriptions	
	Orengedokuto	Dia-Orengedokuto
Da huang (Rhei Radix et Rhizoma)		*
Huang bai (Phellodendri Cortex)	*	*
Huang lian (Coptidis Rhizoma)	*	*
Huang qin (Scutellariae Radix)	*	*
Zhi zi (Gardenia Fructus)	*	*

8.1.2. Herbs proposed as potential alternatives to rhino horn

Nine herbs are proposed as alternatives to rhino horn (Table 8.3). Findings from this study indicate that none of the herbs investigated possess all the TCM functions attributed to rhino horn. Therefore, a combination of two or more herbs may be required to substitute for rhino horn in existing prescriptions containing the animal product. In TCM, rhino horn is mostly used in combination with other medicinal herbs and it is often considered the principal (most important) component in the prescriptions. Again,

combinations of these herbs and appropriate doses would be based on the individual case.

Table 8.3. Nine herbs proposed as potential alternatives to rhino horn

Description of Herb
1. Ban lan gen, root of *Baphicacanthus cusia* Bremek (Acanthaceae)
2. Dan shen, root of *Salvia miltiorrhiza* Bge. (Lamiaceae)
3. Dan zhu ye, aerial part of *Lopthatherum gracile* Brongn. (Poaceae)
4. Jin yin hua, flower bud of *Lonicera japonica* Thunb. (Caprifoliaceae)
5. Lian qiao, fruit of *Forsythia suspensa* Vahl. (Oleaceae)
6. Mu dan pi, root of *Paeonia suffruticosa* Andr. (Paeoniaceae)
7. Sheng di huang, root of *Rehmannia glutinosa* Steud. (Scrophulariaceae)
8. Xuan shen, root of *Scrophularia ningpoensis* Hemsl (Scrophulariaceae)
9. Zi cao, root of *Arnebia euchroma* I.M.Johnst. (Boraginaceae)

8.2. Recommendations for further research work

The anti-bacterial assays conducted in this study have proved useful in identifying herbs with potential pharmacological activities. The determination of the minimum inhibitory concentration of the extracts is necessary to compare them to available anti-bacterial agents.

The inflammatory response is a complex cascade of events and therefore studies are warranted to assess other pharmacological mechanisms through which the plants might mediate anti-inflammatory effects.

There is a need to conduct bioactivity-guided fractionations to determine biologically active fractions and compounds contained within the herbs. Also, the inhibitory NF-κB activities of Trichpsanthis Radix, *Lophatherum gracile* and Sojae Praeparatum Semen were demonstrated for the first time

in the current study and more work should be done to determine the active components.

The epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET) assay developed in this study could be adopted to measure the effect of herbal extracts on EETs (which possess anti-inflammatory properties) and simultaneously give information about possible drug-herb interactions. An effective method would include the identification of the CYPs involved in the reactions.

Contaminants such as metal and pesticide residues can affect some biological activities. Therefore, the study of the effect of contaminants on the biological activity of herb extracts could provide information about their possible antagonist and/or synergistic effects.

More data is required to clarify the contribution of rhino horn to the biological activity of combined horn/herbs extracts.

The main aim of this research was to provide scientific data to support the promotion of alternatives to bear bile and rhino horn. This objective has been achieved and the findings from pharmacological and chemical studies are discussed in this thesis. However, in order to ascertain whether the proposed herbs will be accepted as substitutes to bear bile and rhino horn, the results from this study need to be further discussed with traditional medicine practitioners. The results may then be used by governmental and non-governmental organisations in campaigns against the use of endangered animal products in traditional medicine. In view of the increasing popularity of traditional medicines, there is also a need for continual research to promote the sustainable use of plant species to prevent more species becoming endangered.

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Appendix I. 103 Heat-clearing herbs identified in a literature survey

Plant species	Chinese name and plant part
1. *Actinidia chinensis* P. (Actinidiaceae)	Teng li; root
2. *Adina rubella* H. (Rubiaceae)	Shui yang mei; fruit
3. *Andrographis paniculata* Nees (Acanthaceae)	Chuan xin lian; aerial or whole plant
4. *Anemarrhena asphodeloides* Bunge (Anthericaceae)	Zhi mu; rhizome
5. *Apium graveolens* L. (Apiaceae)	Quin cai; whole plant
6. *Artemisia annua* L. (Asteraceae)	Quin hao; aerial part
7. *Asclepias curassavica* L. (Asclepiadaceae)	Ma li jin; stem and leaf
8. *Aster tataricus* L. (Compositae)	Ziwan; root
9. *Baphicacanthus cusia* Bremek. (Acanthaceae)	Ban lan gen; root
10. *Baphicacanthus cusia* Bremek. (Acanthaceae)	Da qing ye; leaf
11. *Belamcanda chinensis* (L.) DC. (Iridaceae)	She gan; rhizome
12. *Berberis soulieana* Schied. (Berberidaceae)	San ke zhen; root
13. *Blechnum orientale* L. (Blechnaceae)	Guan chong; rhizome
14. *Biota orientalis* (L.) Endl. (Cupressaceae)	Cebaiye; Leafy twigs.
15. *Buddleia officinalis* Maxim. (Loganiaceae)	Mi meng hua; flower
16. *Bupleurum chinense* DC. (Umbelliferae)	Chai Hu, root
17. *Brucea javanica* (L.). Merr (Simaroubaceae)	Ya dan zi; fruit
18. *Cassia tora* L. or *C. obtusifolia* L. (Leguminosae)	Jue ming zi; seed
19. *Celosia argentea* L. (Amaranthaceae)	Qing xiang zi; seed
20. *Centella asiatica* (L.) Urb. (Apiaceae)	Ji xue cao; whole plant
21. *Cimicifuga foetida* L (Ranunculaceae)	Sheng ma; rhizome.
22. *Coix lachryma*-job L. (Gramineae)	Yi yi ren; seed
23. *Commelina benghalensis* L. (Commelinaceae)	Tang shi song; whole plant
24. *Commelina communis* L. (Commelinaceae)	Ya chih tsao; Ya zhi cao; whole plant
25. *Coptis chinensis* Franch. (Ranunculaceae)	Huang lian; rhizome
26. *Crotalaria sessiliflora* L. (Leguminosae)	Ye bai he; whole plant
27. *Curcuma longa* L. (Zingiberaceae)	Yu jin; tubers
28. *Cymbidium ensifolium* Sw. (Orchidaceae)	Jian lan hua; whole plant
29. *Cynanchum stauntoni* (Decne.) (Asclepiadaceae)	Bai qian; root
30. *Dendranthema indicum* (L.) Des Moul. (Compositae)	Ye ju hua; whole plant or leaf
31. *Dendranthema morifolium* (Ramat.) Tzvel. (Compositae).	Ju hua; whole plant or leaf
32. *Dianthus chinensis* L. (Caryophyllaceae)	Qu mai; aerial part
33. *Dryopteris crassirhizoma* Nakai (Pteridophyta)	Guan zhong; rhizome/leaf stalk
34. *Duchesnea indica* (Andr.) Focke. (Rosaceae)	She mei; whole plant
35. *Echinops latifolius* Tausch. (Compositae)	Lou lu; root
36. *Euphorbia humifusa* Willd. (Euphorbiaceae)	Di jin cao; whole plant
37. *Ervatamia divaricata* (L.) (Apocynaceae)	Gou ya hua; whole plant
38. *Fibraurea recisa* Pierre Fl. (Menispermaceae)	Huang teng; stem
39. *Forsythia suspensa* Vahl. (Oleaceae)	Lian qiao, fruit
40. *Fritillaria cirrhosa* D. Don. (Liliaceae)	Chuan bei mu; bulb
41. *Gardenia jasminoides* Ellis (Rubiaceae).	Zhi zi; fruit
42. *Gentiana scabra* Bge. (Gentianaceae)	Long dan; root and rhizome
43. *Glycyrrhiza uralensis* Fisch. (Leguminosae)	Gan cao; root and rhizome
44. *Hedyotis diffusa* Willd (Rubiaceae)	Baihuasheshecao; whole plant
45. *Hemsleya amabilis* Diels (Cucurbitaceae)	Xue dan; root
46. *Houttuynia cordata* Thunb. (Saururaceae)	Yu xing cao; whole plant
47. *Ilex chinensis* Sims. (Aquifoliaceae)	Si ji qing; leaf
48. *Ilex pubescens* Hook & Arn. (Aquifoliaceae)	Bai mao gen; rhizome
49. *Imperata cylindrical* P. Beauv. (Gramineae)	Bai mao gen; rhizome
50. *Iris pallasii* Fischer var. *chinensis* Fisch. (Iridaceae)	Ma lin zi; seed
51. *Lithospermum erythrorhizon* Sieb. & Zucc. (Boraginaceae)	Zi cao; root

Appendix I cont'd.

Plant species	Chinese name and plant part
52. *Livistona chinensis* R. Br. (Palmae)	Kui shu zi; seed
53. *Lobelia chinensis* Lour. (Campanulaceae)	Ban bian lian; whole plant
54. *Lonicera japonica* Thunb. (Caprifoliaceae)	Jin yin hua; flower buds
55. *Lophatherum gracile* Brongn. (Poaceae)	Dan zhu ye; aerial or whole plant
56. *Lycium chinense* Miller (Solanaceae)	Gou qi zi; root
57. *Lysimachia christinae* Hance (Primulaceae)	Jin qian cao; plant part
58. *Menispermum dauricum* DC. (Menispermaceae)	Bei dou gen; rhizome
59. *Mussaenda pubescens* W.T. Ait. f. (Rubiaceae)	Shan-gan-cao; leaf
60. *Nelumbo nucifera* Gaertn (Nelumbonaceae)	He ye; leaf
61. *Ocimum basilicum* L. (Labiatae)	Guang ming zi; fruit
62. *Paeonia lactiflora* Pall (Paeoniaceae)	Chi shao yao; root
63. *Paeonia suffruticosa* Andr. (Paeoniaceae)	Mu dan pi; root bark
64. *Patrinia scabiosaefolia* Link (Valerianaceae)	Bai jiang; whole plant or rhizome
65. *Perilla frutescens* (L.) Britton var. crispa Decne. (Labiatae)	Zi Su; tzu-su; tzu-su-yeh; leaf
66. *Peucedanum praeruptorum* Dunn. (Umbelliferae)	Qian hu; root
67. *Phellodendron amurense* Ruprecht (Rutaceae)	Huang bai; root bark
68. *Phragmites communis* Trin. (Gramineae)	Lu gen; rhizome
69. *Picrorhiza kurrooa* Royle (Scrophulariaceae)	Hu huang lian; rhizome
70. *Pleioblastus amarus* Keng (Gramineae)	Ku zhu ye; leaf
71. *Polygonum chinense* L. (Polygonaceae)	Huo tan mu cao; whole plant
72. *Polygonum cuspidatum* Sieb. & Zucc. (Polygonaceae)	Hu zhang; rhizome
73. *Populus euphratica* Oliv. (Salicaceae)	Hu tong lei; resin
74. *Portulaca oleracea* L. (Portulacaceae)	Ma chi xian; stem
75. *Prunella vulgaris* L (Labiatae).	Xia ku cao; spike or whole plant
76. *Pueraria lobata* (Willd.) Ohwi (Fabaceae)	Ge gen; root
77. *Pulsatilla chinensis* (Bunge) Regel (Ranunculaceae)	Bai tou weng; root
78. *Rabdosia rubescens* (Hemsl.) (Labiatae)	Dong ling cao; whole plant
79. *Ranunculus ternatus* Thunb. (Ranunculaceae)	Mao zhua cao; root
80. *Rauvolfia verticillata* Bail. (Apocynaceae)	Luo fu mu; root
81. *Rehmannia glutinosa* Steud. (Scrophulariaceae)	Sheng di huang; root
82. *Rheum palmatum* L. (Polygonaceae)	Da huang; root and rhizome
83. *Salvia miltiorrhiza* Bge. (Lamiaceae)	Dan shen; root
84. *Sanguisorba officinalis* L. (Rosaceae)	Di yu; root and rhizome
85. *Sargentodoxa cuneata* Rehd. & Wils. (Lardizabalaceae)	Hong teng; stem
86. *Saxifraga stolonifera* Meerb. (Saxifragaceae)	Hu er cao; leaf
87. *Schizonepeta tenuifolia* Briq.(Labiatae)	Jing jie; aerial part
88. *Scrophularia ningpoensis* Hemsl. (Scrophulariaceae)	Xuan shen; root
89. *Scutellaria baicalensis* Georgi (Labiatae)	Huang quin; root
90. *Sedum sarmentosum* bunge (Crassulaceae)	Chui pen cao; whole plant
91. *Senecio scandens* Buch.-Ham (Compositae)	Qian li guang; aerial or whole plant
92. *Solanum nigrum* L. (Solanaceae)	Long kui; roots and seeds
93. *Sophora angustifolia* Siebold & Zucc. (Leguminosae)	Ku shen; root
94. *Sophora subprostrata* Chun & T.C. Chen (Leguminosae)	Shan dou gen; root
95. *Stellaria dichotoma* L. var lanceolata Bge. (Caryophyllaceae)	Yin chai hu; root
96. *Swertia pseudochinensis* Hara (Gentianaceae)	Dang yao; whole plant
97. *Taraxacum mongolicum* Hand.-Mazz. (Compositae)	Pu gong ying; whole plant
98. *Tetrastigma delavayi* G. (Vitaceae)	Wu lian mei; whole plant and root
99. *Trichosanthes kirilowii* Maxim. (Cucurbitaceae)	Gua lou; fruit
100. *Tussilago farfara* L. (Compositae)	Tung hua; flower stem
101. *Uncaria ryhnchophylla* Miq. (Rubiaceae)	Gou teng; stem
102. *Usnea diffracta* Vain. (Lichens)	Song luo; mycelia
103. *Viola yedoensis* Makino (Violaceae)	Zi hua ti ting; fruit

Appendix II. Herbal alternatives to bear bile (IFAW report, 1994).

Plant species	Plant part used Comments have been inserted
1. *Lobelia chinensis* Lour.	Whole plant (similar to 6)
2. *Costus speciosus* (Koenig) Smith	Bulb
3. *Curcuma zedoaria* (Berg.) Rose	Root and Stem
4. Radix et Rhizoma Rhei	Root and rhizome
5. Radix Scutellaria	Root
6. Herba Lobelia Chinensis	Whole plant (similar to 1)
7. *Smilax china* L.	Rhizome
8. *Andrographis paniculata* (Burm.f) Nees	Whole plant
9. Herba Saururi Chinensis	Whole plant
10. *Sarcandra glabra* (Thunb.) Nakai	Whole plant (similar to 24)
11. *Scutellaria barbata* Don	Whole plant (similar to 54)
12. *Cycas revoluta* Thunb.	Leaves, flowers, seeds and roots
13. Herba Taraxaci	Whole plant
14. Herba Cirsii Japonici	Arial parts
15. *Selaginella doederleinii* Hieron	Whole plant
16. *Adiantum flabellutatum* Linn.	Whole plant
17. *Impatiens balsamina* Linn.	Seeds and flowers
18. *Osbeckia chinensis* Linn.	Whole plant
19. *Paris chinensis* Fr.	Roots
20. Radix Rhapontici	Roots
21. Herba Sedi Aizoon	Whole plant
22. *Chrysanthemum indicum* L.	Whole plant or flowers
23. Herba Andrographitis	Aerial parts
24. Herba Sarcandrae	Whole plant (similar to 10)
25. Herba Salvia Plebeiae	Aerial parts
26. Herba Hedyotis Diffusae	Whole plant (similar to 40)
27. Herba Houttuyniae	Aerial parts
28. Folium Ilieis Chinensis	Leaves
29. *Dioscorea bulbifera* L.	Tuber and auxillary sprouts (similar to 35)
30. Folium et Ramulus Cephalotaxi	Twig and leaf
31. Cortex Phellodendron	Bark
32. Herba Verbenae	Arial parts
33. Folium Hibisci Mutabilis	Leaves
34. *Catharanthus roseus* (L.) G. Don	Whole plant (similar to 53)
35. Rhizoma Dioscorae Bulbiferae	Tuber (similar to 29)
36. *Melothria heterphylla* (Lour.) Cogn.	Whole plant or tubers
37. *Camptotheca acuminata* Decne	Fruits, leaves, branches, bark and root
38. *Livistona chinensis* R. Br.	Seeds , root and leaf
39. *Gardenia jasminoides* Ellis	Fruits and roots
40. *Hedyotis diffusa* Wild.	Whole plant (similar to 26)
41. *Hedyotis tenelliflora* Blume.	Whole plant
42. *Duchesnea indica* (Andr.) Focke	Whole plant (similar to 52)
1. 43. *Hedyotis corymbosa* (L.) Lamk.	Whole plant
44. *Hedyotis auricularia* L.	Whole plant
45. *Passiflora foetida* L.	Whole plant and fruits
46. Caulis Hederae Sinensis	Stem
47. *Acanthus ilieifolius* Linn.	Root
48. Radix Paeoniae Sinjiagenensis	Root
49. *Smilax glabra* Roxb.	Root
50. *Xanthium sibricum* Patrin.	Whole plant or fruit
51. *Houttuynia cordata* Thunb.	Whole plant
52. Herba Duchesneae Indicae	Whole plant (similar to 42)
53. Herba Catharanthi Rosei	Whole plant (similar to 34)
54. Herba Scutellaria Barbatae	Whole plant (similar to 11)

Appendix III. The effects of changes in the flavonoid structure on the UV absorption maxima, band I and band II.

Band I	Band II
Conjugation between A and B rings	
Lack of conjugation between A and B rings, i.e. no double bond between carbon-2 and carbon-3 of the flavonoid skeleton: low intensity in band I (for example isoflavones, flavones (dihydroflavones) and dihydroflavonols (Grayer, 1989; also see Appendix V).	Lack of conjugation between A and B rings: high intensity in band II (for example isoflavones, flavones (dihydroflavones) and dihydroflavonols (Grayer, 1989; also see Appendix VI). Conjugation between A and B rings: Low intensity in band II
Effect of changes in the substitution of the A-, B- and C-rings	
Changes in the substitution of the B- and C-rings are more pronounced (Markham, 1982; Harborne *et al.*, 1975). For example Flavones: 304-350 nm (Harborne *et al.*, 1975) Flavonols: 352-385 nm (Harborne *et al.*, 1975); (OH on 3 position; C-ring)	Changes in the substitution of the A-ring more pronounced (Markham, 1982; Harborne *et al.*, 1975). For example 7-Hydroxyflavone 252 nm (Harborne *et al.*, 1975) 5,7-Dihydroxyflavone 268 nm (Harborne *et al.*, 1975) 5,6,7-Trihydroxyflavone 274 nm (Harborne *et al.*, 1975) 5,7,8-Trihydroxyflavone 281 nm (Harborne *et al.*, 1975) Less affected by changes in the B- and C-rings. For example, Flavone 250-280 nm; Flavonols: 250-270 nm (Harborne, 1989). However, 3'4'-dihydroxylated flavones (B-ring substitution) shows two peaks (or a peak with a shoulder) in this area, while 4'-hydroxylated flavones only shows peak in band I (Harborne *et al.*, 1975)
Effect of additional oxygenation (especially hydroxylation)	
Shift to longer wavelengths (bathohromic), for example, 3,5,7-triOH (galangin) 359 nm (Harborne *et al.*, 1975) 3,5,7,4'-tetraOH (kaempferol) 367 nm (Harborne *et al.*, 1975) 3,5,7,3',4'-pentaOH (quercetin) 370 nm (Harborne *et al.*, 1975) 3,5,7,3',4',5'-hexaOH (myricetin) 374 nm (Harborne *et al.*, 1975)	Shift to longer wavelengths (bathohromic) (examples above; under '*Effect of changes in the substitution of the A- and B-rings*')
Absence of hydroxyl groups	
Evidenced by weak intensity (Harborne *et al.*, 1975)	Evidenced by weak intensity (Harborne *et al.*, 1975)
Methylation or glycosylation on the flavonoid nucleus	
Shifts to shorter wavelengths (hypsochromic). For example substitution of a 4'-hydroxyl group: 3-10 nm shift (Markham, 1982) substitution of a 5-hydroxyl group: 5-15 nm shift (Markham, 1982)	Especially of 3, 5, 7 and 4' OHs: Shifts to shorter wavelengths (hypsochromic). E.g. substitution of a 5-hydroxyl group: 5-15 nm shift (Markham, 1982) substitution of a 3-hydroxyl group:12-17 nm shift (Markham, 1982) substitution of a 3-hydroxyl group: 22-25 nm shift on substitution of a 5-deoxyflavonols (Harborne *et al.*, 1975; Markham, 1982) Similar substitution at other sites in the molecule has little effect on the UV spectra
Acylation	
Acetylation (CH_3CO-) tends to nullify the effect of a phenolic OH group on the spectrum and is therefore a good technique for locating alkoxy groups (Harborne *et al.*, 1975; Markham, 1982). For example, methylation of hydroxyflavones in the 4'-position gives UV spectra approximating that of 4'-methoxyflavone (320 nm) (Harborne *et al.*, 1975). Similarly, The presence of cinnamic acid acyl group can be detected by an absorption band at *c.* 320 nm in flavonoids that lack significant absorption in this region (e.g. anthocyanins) (Markham, 1982)	Acylation (addition of RCO-) such as acetylation (CH_3CO-) tends to nullify the effect of a phenolic OH group on the spectrum

Appendix IV. The effects of changes in the flavonoid structure on chromatographic retention time (Summarized from Ferreres et al., 1989).

Flavonoid structure	Effect on retention time (in reversed phase HPLC)
FLAVONOID AGLYCONES	
Internal hydrogen (H) bonding	Decreases the capacity of the compound to interact with the solvent (mobile phase) and thereby increases retention time (Rt)
Carbonyl group (C=O) at the 4-position	The C-4 carbonyl group possess the strongest H-bond in the structure due to a partial negative charge caused by resonance
Internal hydrogen between C=O at the 4-position and hydroxyl (OH) at the 5-position	Increase in Rt. A strong internal H-bond is formed between OH group at C-5 and the C=O group at C-4, reducing the interaction between the C=O and the solvent. This leads to an increase in Rt values for 5-hydroxy-flavonoids compared their counterparts not possessing a free OH group
Internal hydrogen between OH groups and C-6 and C-5	5,6-Dihydroxyflavonoids elute with shorter Rt than corresponding isomers. Internal H-bonding between the OH groups at C-6 and C-5 decreases interaction between the C-5 OH group and the 4-keto group and so decreases Rt. This is the only case in which an internal H-bond between two OH groups decreases Rt.
Free C-5 OH compared to C-5 methoxy (OMe)	5-hydroxyflavones elute with shorter Rt than 5-methoxy counterparts since the latter is more hydrophobic
Internal hydrogen between C=O at the 4-position and hydroxyl (OH) at the 3-position	H-bonding between the carbonyl group and C-3 OH is comparatively (to C-5 OH) weaker
OH groups at positions other than 3 or 5	Reduction of Rt by 1.43 to 1.46 minutes, if there is no OH group already present at the *ortho* (*o*) position to the position considered. If an *o*-OH group is already present the decrease in Rt is only 0.86-3.3
OH/Ome ratio	The lower the OH/OMe ratio, the higher the Rt
H-bonding between OH groups at 3' and 4'	Increases Rt compared to compounds bearing the same substitution patterns but with OH/OMe groups. Although, the methylation of a OH group should reduce a compound's polarity and increase Rt, the elimination of the H-bonding between 3' and 4' has a greater effect. For example, 5,3',4'-trihydroxy-6,7,8-trimethoxyflavone and 5,3',4',-trihydroxy-6,7-dimethoxyflavove elute with higher Rt than 5,8,4'-trihydroxy-6,7,3'-trimethoxyflavone and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone, respectively. Also, 3,5,7,4'-tetrahydroxy-3'-methoxyflavone (isohamnetin) and 3,5,7,3',4'-pentahydroxyflavone (quercetin) have similar Rt

Appendix IV. cont'd.

Flavonoid structure	Effect on retention time (in reversed phase HPLC)
OMe group on the B-ring (structure and size effect)	Reduces Rt. The smallest molecules can interact more easily with the C18 branches of the stationary phase (SP). Thus, flavones bearing a single OMe group on the B-ring interact more strongly with the SP than their counterparts bearing two OMe groups on the ring (although the introduction of OMe grous increases lipophilicity)
OMe group on the B-ring (structure and size effect)	Reduces Rt. 3,5,6,7-tetramethoxyflavone < 5,6,7-trimethoxyflavone < 5,7-dimethoxyflavone
OH-group at C-4' (B-ring)	The size effect is not observed when OH is present at C-4', possibly owing to H-bond formation, which is the main effect in these compounds, is less affected by the molecular size than the hydrophobic effect
OMe group on the A-ring (structure and size effect)	Dramatically increases Rt (depending on the substitution pattern).
FLAVONOID GLYCOSIDES	
Glycosylation	As a general rule, Rt is inversely correlated with increasing glycosylation, and the position of the glycosylation has a significant effect on the mobility Order of elution Triglycosides > diglycosides > monoglycosides
Type of sugar	The contribution of various types of sugars to the hydrophobic interaction decreases from hexoses through to pentoses to methylpentoses. Glucosides > arabinosides > rhamnosides. A diglycoside with only glucose moieties will elute sooner than one or two rhamnose units
Flavonoid sulphates	Sulphate residue on a OH of a flavonoid aglycone or glycoside increases its polarity and decreases its Rt
Monosulphates and disulphates	Generally flavonoid disulphates elute before the respective monosulphates Flavonoid monosulphates elute with a comparatively sharper peak due to the ionisation of the sulphate group
Introduction of a second sulphate	At the 6-position in 7-monosulphates: decreases the Rt by more than 2 minutes. At the 4'-position in 7-monosulphates: decrease in Rt depends on the substitution pattern on the B-ring: if a methyl ether already present at the 3'-osition then the decrease is about 1 minute. Flavones with a monosubstituted B-ring, the decrease is about 4 minutes

Appendix V. Ultraviolet (UV) maxima of classes of phenolic compounds in methanol.

Class and basic skeleton	UV (MeOH) λmax, nm	Reference
Benzoquinones (C6)	260-290, 375-410	Dey and Harborne, 1989
Phenols (C6)	266-295	Dey and Harborne, 1989
Phenolic acids (C6-C1)	235-305	Dey and Harborne, 1989
Hydroxycinnamic acids (C6-C3)	227-245, 310-332	Dey and Harborne, 1989
Hydroxycoumarins (C6-C3)	210, 250-260, 280-303, 312-351	Dey and Harborne, 1989
Napthoquinones (C6-C4)	220-250, 250-290, 330-340, 400-430	Dey and Harborne, 1989
Xanthones (C6-C1-C6)	230-245, 250-265, 305-330, 340-400	Dey and Harborne, 1989
Stilbenes (C6-C2-C6)	300-310, 320-330	Dey and Harborne, 1989
Phenanthrenes (C6-C2-C6)	265-270, 272-280, 300-306, 310-315	Dey and Harborne, 1989
Anthraquinones (C6-C2-C6)	220-230, 252-260, 267-279, 430-450	Dey and Harborne, 1989
Flavonoids (C6-C3-C6)		
1. Chalcones	240-260, 365-390 230-270 (low intensity), 340-390	Dey and Harborne, 1989 Markham, 1982
2. Flavanones	c. 225, 275-290, 310-330	Dey and Harborne, 1989
and dihydroflavonols	275-295, 300-330s	Markham, 1982
3. Flavones, biflavones	250-270, 330-350 250-280, 310-335	Dey and Harborne, 1989 Markham, 1982
4. Flavonols	250-270, c, 300, 350-390	Dey and Harborne, 1989
3-OH substituted	250-280, 330-360	Markham, 1982
3-OH free	250-280, 350-385	Markham, 1982
5. Anthocyanins	267-275, 475-545 (in ethanol)	Dey and Harborne, 1989
anthocyanidins	270-280, 465-560	Markham, 1982
6. Aurones	230-270 (low intensity), 380-430	Markham, 1982

Appendix V. cont'd.

Flavonoid subgroup	UV (MeOH) λmax, nm	Reference
7. *Isoflavonoids (C6-C3-C6)*		
7a. Isoflavones	245-275, (310-330s low intensity) 255-265, 310-330	Markham, 1982 Dey and Harborne, 1989
5-deoxy-isoflavones	*c.* 320 peak	Markham, 1982
7b. Isoflavanones	270 and 310 nm	Dey and Harborne, 1989
7c. Pterocarpans		
no additional aromatic rings	281 and 287	Dey and Harborne, 1989
8,9-disubstituted	300-310	Dey and Harborne, 1989
Pterocarpenes	335 and 353	Dey and Harborne, 1989
7d. Coumestans	340-350	Dey and Harborne, 1989
7e. Rotenoids	280-300	Dey and Harborne, 1989
Dehydrorotenoids	275-280	Dey and Harborne, 1989
6-Oxodehydrorotenoids	300-320, 260-270, 290-300	Dey and Harborne, 1989
7f. Isoflav-3-enes	320-325	Dey and Harborne, 1989
7g. 3-Arylcoumarins	340-360	Dey and Harborne, 1989
3-Aryl-4-hydroxycoumarins	340-360	Dey and Harborne, 1989
7h. 3-Arylbenzofuran	320 and 335	Dey and Harborne, 1989
7i. Isoflavanquinones	265-270 (360-400 low intensity)	Dey and Harborne, 1989

Appendix VI. Information concerning the Chinese and Latin names of plants used in this study as well as the voucher numbers of the different samples studied.

TCM names	Species	Family	Samples	Reference samples
Ban lan gen Isatidis Radix	*Isatis indigotica* Fort. *I. tinctoria* L. *Baphicacanthus cusia* Bremek	Cruciferae Acanthaceae	(1) BI 9862 (MY1, batch 5672) (2) BI 10251 (MY2, batch 5672) (3) BI 9894 (KH)	(1) BI 9941, TCMK 57 (2) BI 11550, EBC no. 81496 (3) BI 11552, EBC no. 81508 (1-3: *Isatis indigotica* Fort)
Chi shao yao Paeoniae Rubra Radix	*Paeonia vetchii* Lynch *P. lactiflora* Pall. *P. obvota* Maxim.	Paeoniaceae	(1) BI 9863 (MY1, batch 5704) (2) BI 10258 (MY2, batch 5704) (3) BI 9895 (EW)	(1) B1 9942, TCMK 66, *Paeonia lactiflora* Pall (2) B1 9943, TCMK 66, *Paeonia vetchii* Lynch
Chuan xin lian Andrographitis Herba	*Andrographis paniculata* (Burm.f) Nees	Acanthaceae	(1) BI 9864 (batch 5456) (2) BI 10242 (batch 5456) (3) BI 9896	(1) B1 9944, TCMK 19 (2) B1 9945, TCMK 47
Dan shen Salviae Miltiorrhizae Radix	*Salvia miltiorrhiza* Bunge	Lamiaceae	BI 10718 (batch 5730)	(1) BI 9938, TCMK 4 (2) BI 10707, TCMK 259 (3) BI 10708, EBC no. 81016 (market sample)
Dan zhu ye Lophatheri Herba	*Lophatherum gracile* Brongn.	Poaceae	(1) BI 9866 (MY1) (2) BI 10254 (MY2) (3) BI 9898 (KH)	(1) BI 11574, EBC 81229 (2) BI 11575, EBC 81239 (3) BI 11576, EBC 81300 (4) BI 11577, EBC 81289 (5) BI 11578, EBC 81269
Gan cao Glycyrrhizae Radix	*Glycyrrhiza uralensis* Fisch. *G. inflata* Batal. *G. glabra* L.	Leguminosae	(1) BI 9867 (MY1, batch 4813) (2) B1 10256 (MY2, batch 4812) (3) BI 9899 (KH)	(1) BI 9969, TCMK 99, *Glycyrrhiza* species. (2) BI 11527, EBC 81409 (3) BI 11528, EBC 81453 (2 & 3 *Glycyrrhiza uralensis* Fisch.)
Huang bai Phellodendri Cortex	*Phellodendron amurense* Rupr. *P. chinensis* Schneid.	Rutaceae	BI 10723 (MY2, batch 5095)	BI 10111, TCMK 67 *Phellodendron amurense* Rupr.
Huang lian Coptis Rhizoma	*Coptis chinensis* Franch. *C. deltoidea* Cheng et Hsiao *C. teetoides* C. Y. Cheng *C. omeiensis* C. Y. Cheng	Ranunculaceae	(1) BI 9868 (MY1, batch 5791) (2) BI 10260 (MY2, batch 5791) (3) BI 9900 (KH)	BI 8791, TCMK 126, *Coptis* sp.

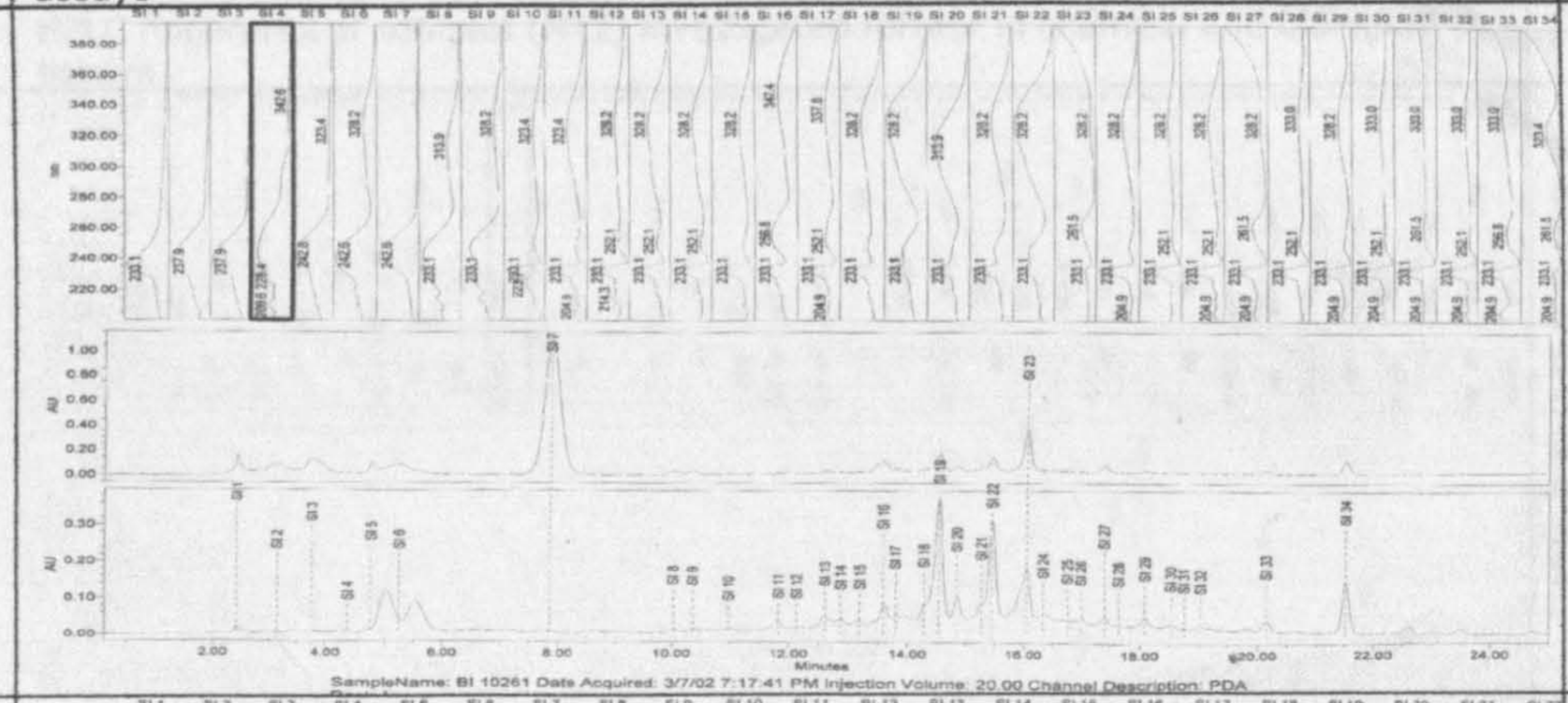
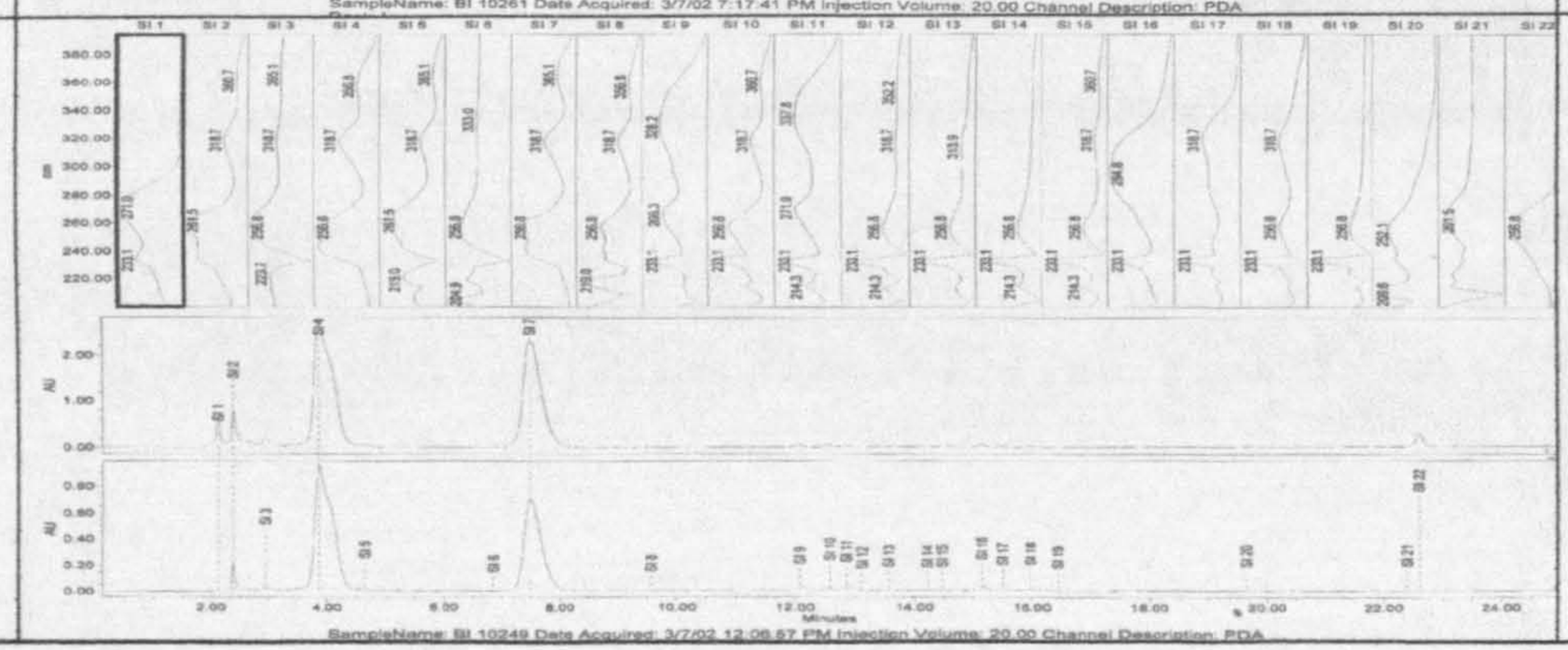
Table VI. cont'd.

TCM names	Species	Family	Samples	Reference samples
Huang qin Scutellariae Radix	*Scutellaria baicalensis* Georgi *S. viscidula* Bge. *S. amoena* Wright *S. ikonnikovii* Juz. *S. rehderiana* Diels *S. hypericifolia* H.Lév. *S. liklangensis* Diels	Labiatae	(1) BI 9869 (MY1, batch 5736) (2) BI 10255 (MY2, batch 5736) (3) BI 9901(KH)	BI 9948, TCMK 7, *Scutellaria baicalensis* Georgi
Jie geng Platycodi Radix	*Platycodon grandiflorum* (Jacq.) A.DC.	Campanulaceae	(1) BI 9878, (MY1, batch 5708) (2) BI 10252, (MY2, batch 5708 (3) BI 9902 (KH)	BI 10072, TCMK 90
Jin yin hua Lonicerea Herba	*Lonicera japonica* Thunb. *L. confusa* DC. *L. dasystyla* Rehd. *L. hypoglauca* Miq.	Caprifoliaceae	(1) B1 9870 (MY1) (2) BI 10253 (MY2) (3) BI 9903 (KH)	BI 11572, EBC 81597, *Lonicera japonica* Thunb
Lian qiao Forsythia Fructus	*Forsythia suspensa* Vahl. *F. viridissima* Lindl. *F. koreana* Nakai	Oleaceae	(1) BI 9871 (MY1, batch 5374) (2) BI 10257 (MY2, batch 5374) (3) BI 9904 (KH)	BI 9949, TCMK 49, *Forsythia suspensa* Vahl.
Mai men dong Ophiopogonis Radix	*Ophiopogon japonicum* (Thunb.) Ker-Gawl *O. platyphyllus* Merr. *O. spicatus* Hook	Liliaceae	BI 10758, (MY2, batch D148)	BI 10074, EBC 80221, *Ophiopogon* species
Mu dan pi Moutan Radicis Cortex	*Paeonia suffruticosa* Andr.	Paeoniaceae	(1) BI 9872 (batch 5090, MY1) (2) BI 10259 (batch 5090, MY2) (3) BI 9905 (EW)	BI 9975, TCMK 136
Sheng di huang Rehmanniae Radix	*Rehmannia glutinosa* (Gaertn.) Steud.	Scrophulariaceae	(1) BI 9873, (MY1, batch 5722) (2) BI 10262, (MY2, batch 5722) (3) BI 9906 (KH)	(1) BI 8800, TCMK 92 (1 of 2) (2) BI 8799, TCMK 92 (3 of 3)
Xuan shen Scrophulariae Radix	*Scrophularia ningpoensis* Hemsl. *S. buergeriana* Miq. *S. oldhami* Oliver	Scrophulariaceae	(1) BI 9875 (MY1, batch 5738) (2) BI 10263 (MY2, batch 5738) (3) BI 9907 (KH) (4) BI 9890 (KH, powder)	(1) BI 9950, TCMK 53 (2) B1 11689, TCMK 252 (3) B1 11691, TCMK 252 (4) B1 11692, TCMK 252 (1-4:*Scrophularia ningpoensis*)

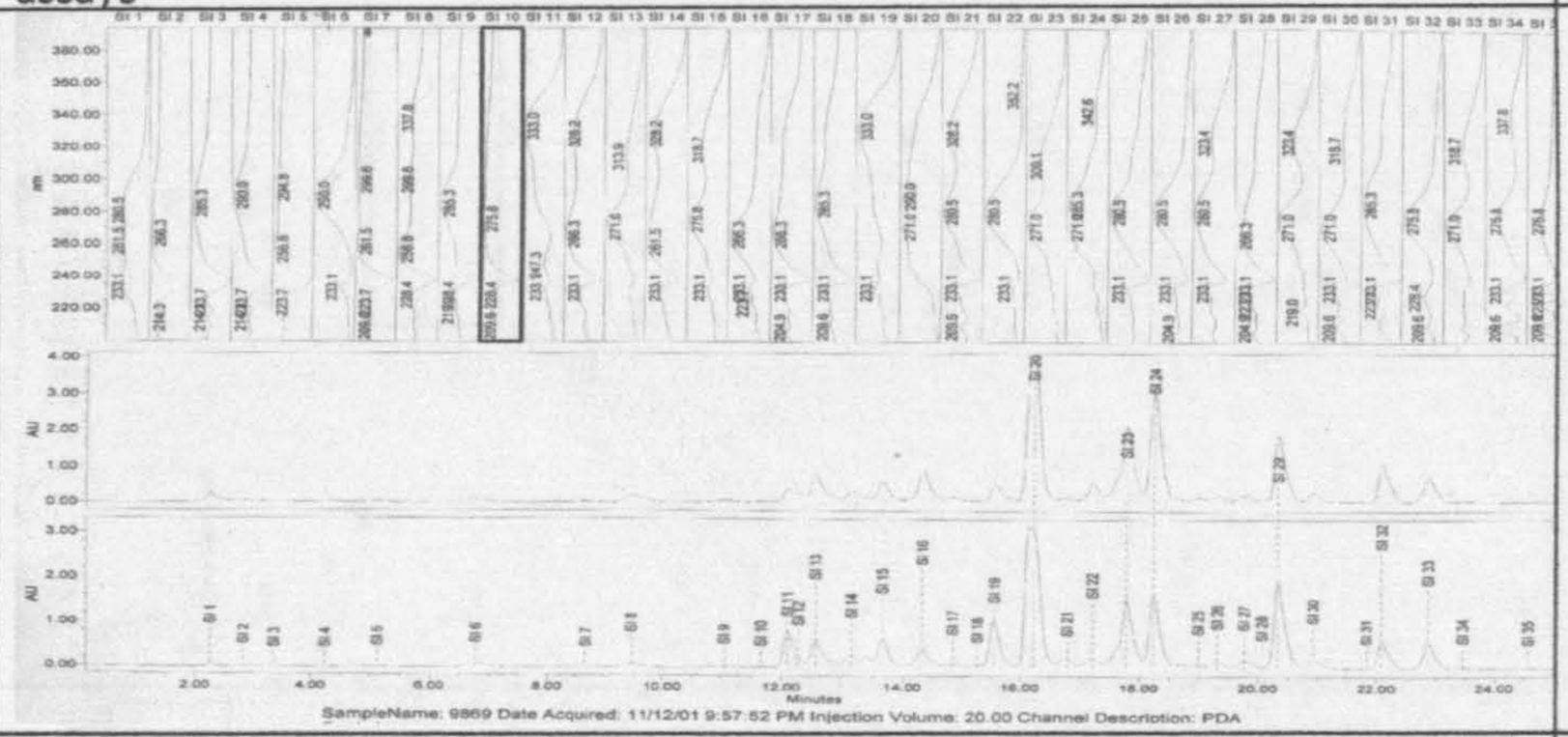
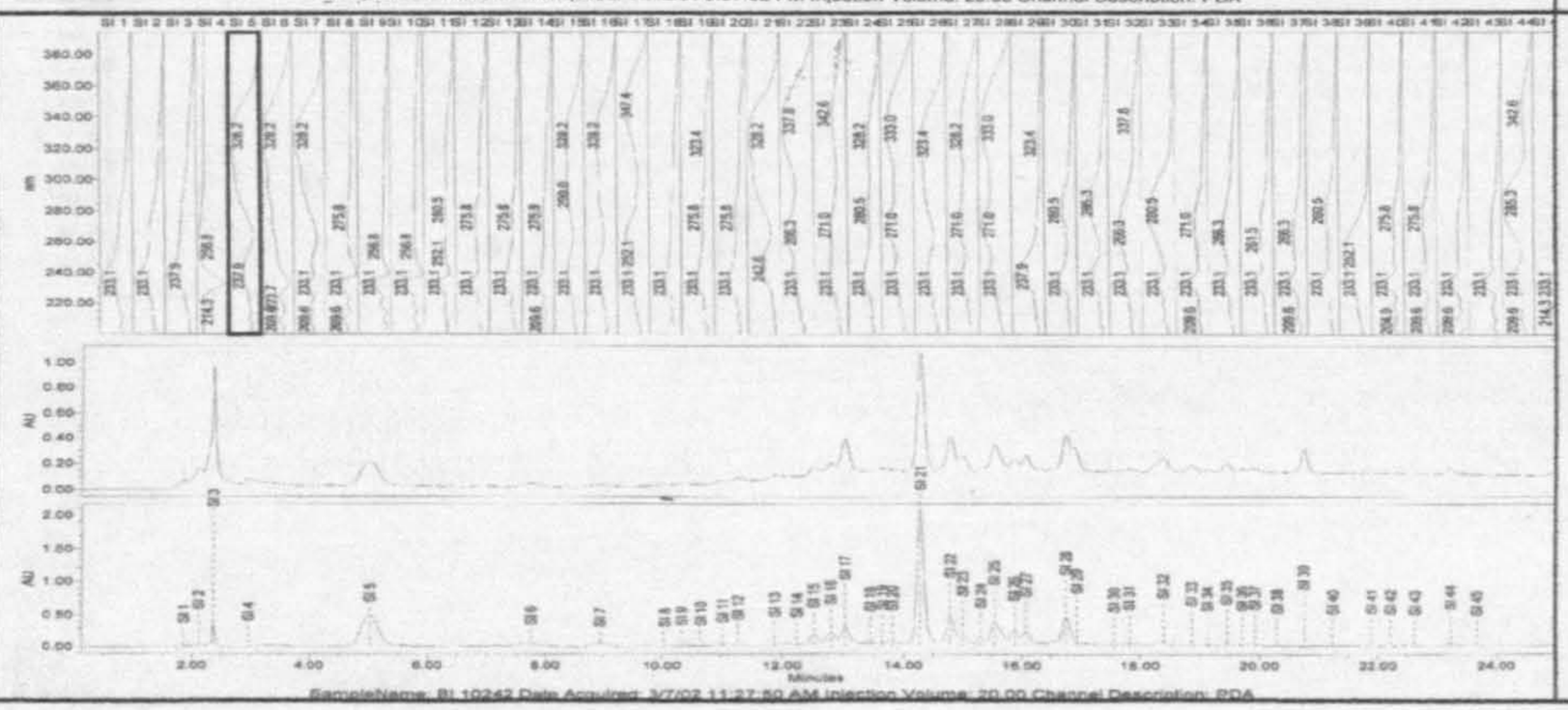
Table VI. cont'd.

TCM names	Species	Family	Samples	Reference samples
Zhi mu Anemarrhena Rhizoma	*Anemarrhena asphodeloides* Bunge.	Anthericaceae	(1) BI 9876 (MY1, batch 5772) (2) BI 10249 (MY2, batch 5772) (3) BI 9908 (KH)	BI 9014, TCMK 69
Zhi zi Gardenia Fructus	*Gardenia augusta* Merr. *G. jasminoides* Ellis	Rubiaceae	(1) BI 9877 (MY1, batch 5376) (2) BI 10261 (MY2, batch 5376) (3) BI 9909 (KH)	(1) BI 9951, TCMK 46, *G. augusta* (2) BI 11524, EBC 81354, *G. jasminodes*
Zi cao Lithospermi Anebiae seu Radix	*Lithospermum erythrorhizon* Sieb. & Zucc. *Arnebia euchroma* I.M.Johnst. *Macrotomia euchromon* Paulsen	Boraginaceae	BI 10724 (MY2)	(1) BI 11378, EBC 81467, *Arnebia euchroma* (2) BI 11379, EBC 81435, *Arnebia euchroma* (3) BI 11565, EBC 81213, *Lithospermum erythrorhizon*
Chang pu Acori Graminei Rhizoma	*Acorus gramineus* [Soland.]	Acoraceae	BI 10741 (MY2)	No reference sample
Da huang Rhei Radix et Rhizoma	*Rheum palmatum* L. *R. tanguticum* Maxim. ex Balf. *R. officinale* Baill. *R. coreanum* Nakai *R. undulatum* L	Polygonaceae	BI 10732 (MY2)	No reference sample
Lian zi xin Nelumbinis Nucifera Plumula	*Nelumbo nucifera* Gaertn.	Nelumbonaceae	BI 10757 (MY2)	No reference sample
Tian hua fen Trichosanthis Radix	*Trichosanthes Kirilowii* Maxim. *T. japonica* Regel	Cucurbitaceae	BI 10721 (MY2)	No reference sample
Dan dou chi Sojae Praeparata semen	*Glycine max* Merr.	Leguminosae	BI 10734 (MY2)	No reference sample

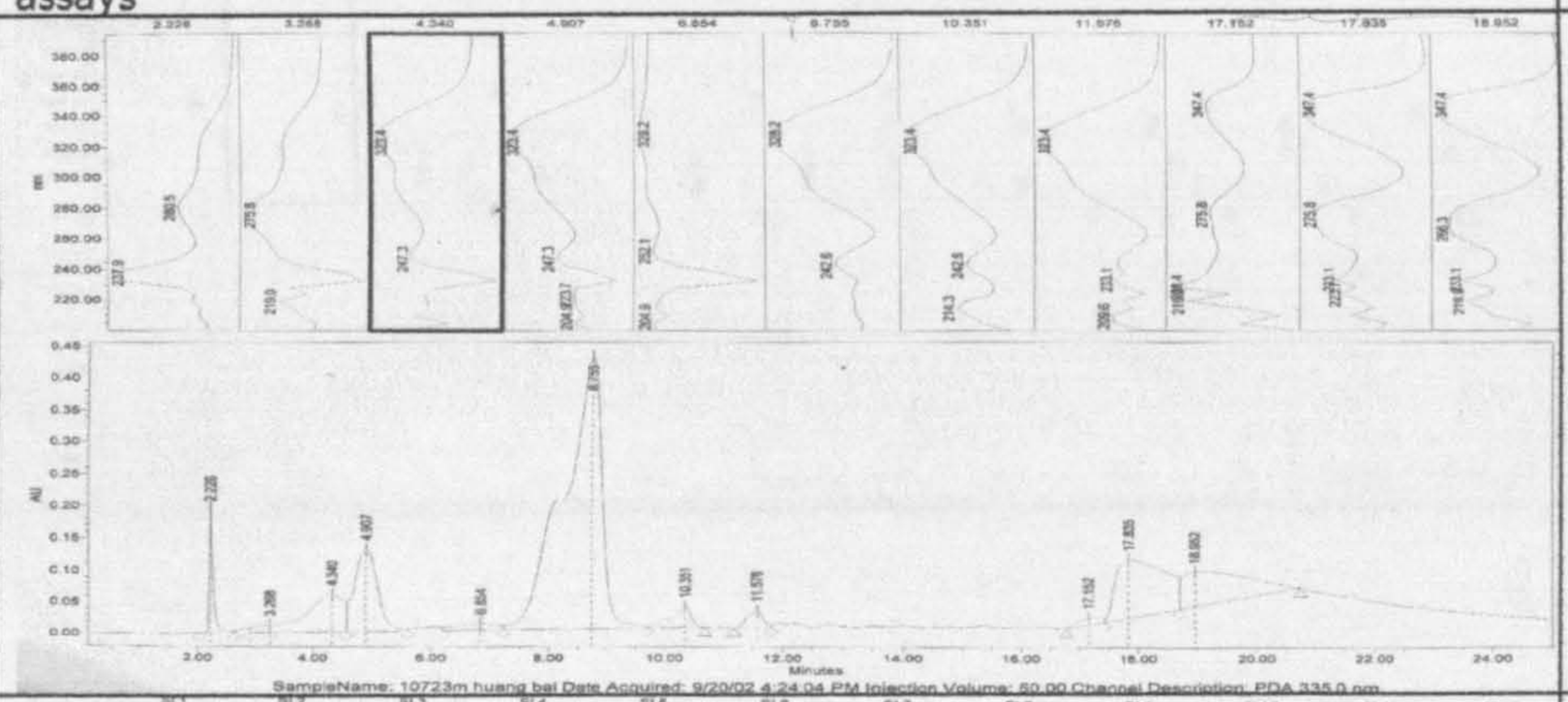
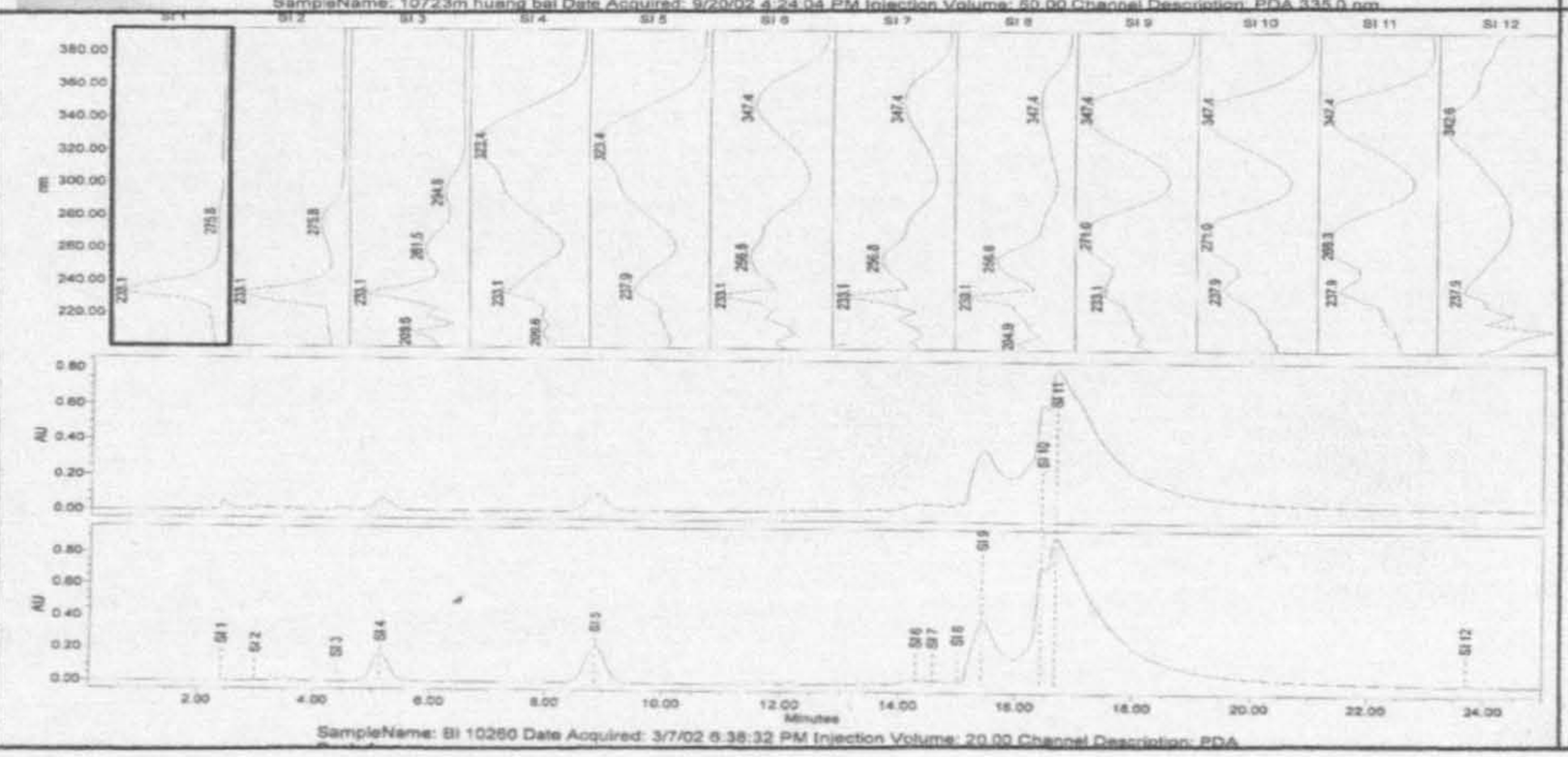
Appendix VIIa. Authentication results for the bear bile project: information about the Chinese and Latin names of plants used in this study, their voucher numbers and chemical profiles, as well as identified compounds

TCM names and voucher number	Plant species and family	HPLC fingerprint of samples (MY2) investigated further in chemical and biological assays	Compounds
Zhi zi, Gardenia Fructus (BI 10261)	*Gardenia jasminoides* Ellis (Rubiaceae)		None identified
Zhi mu, Anemarrhena Rhizoma (BI 10249)	*Anemarrhena asphodeloides* Bge. (Liliaceae)		SI 16: stilbene

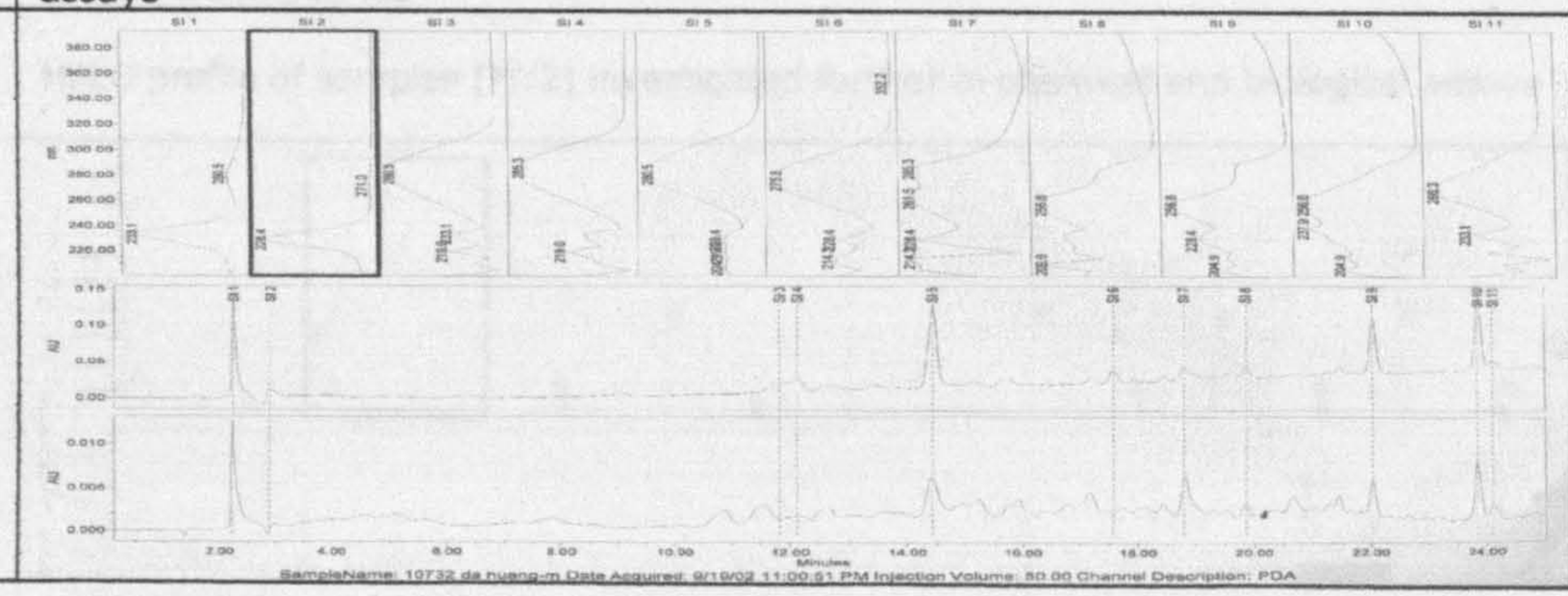
Appendix VIIa cont'd.

TCM names and voucher number	Plant species and family	HPLC fingerprint of samples (MY2) investigated further in chemical and biological assays	Compounds
Huang qin, Scutellariae Radix (BI 10255)	*Scutellaria baicalensis* Georgi (Labiatae)		SI 4: flavanone glycoside SI 11 & 19: verbascoside-type caffeic acid derivative SI 13 & 15: flavones without B-ring hydroxyl groups SI 32: wogonin SI 33: oroxylin A
Chuan xin lian, Andrographitis Herba (BI 10242)	*Andrographitis paniculata* Nees (Acanthaceae)		SI 7, 16 & 21: caffeic acid derivatives SI 17: luteolin 7-glycoside SI 22: apigenin 7-glycoside SI 25, 27 and 28: apigenin glycoside

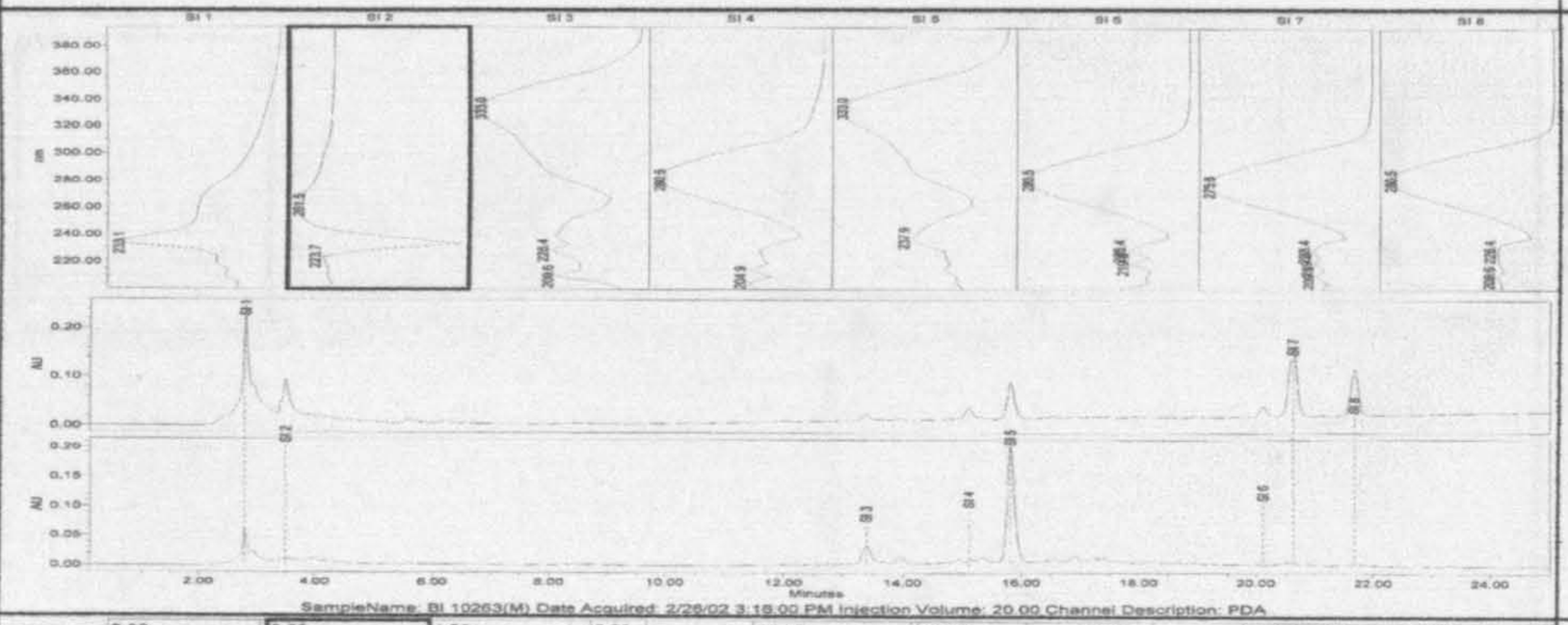
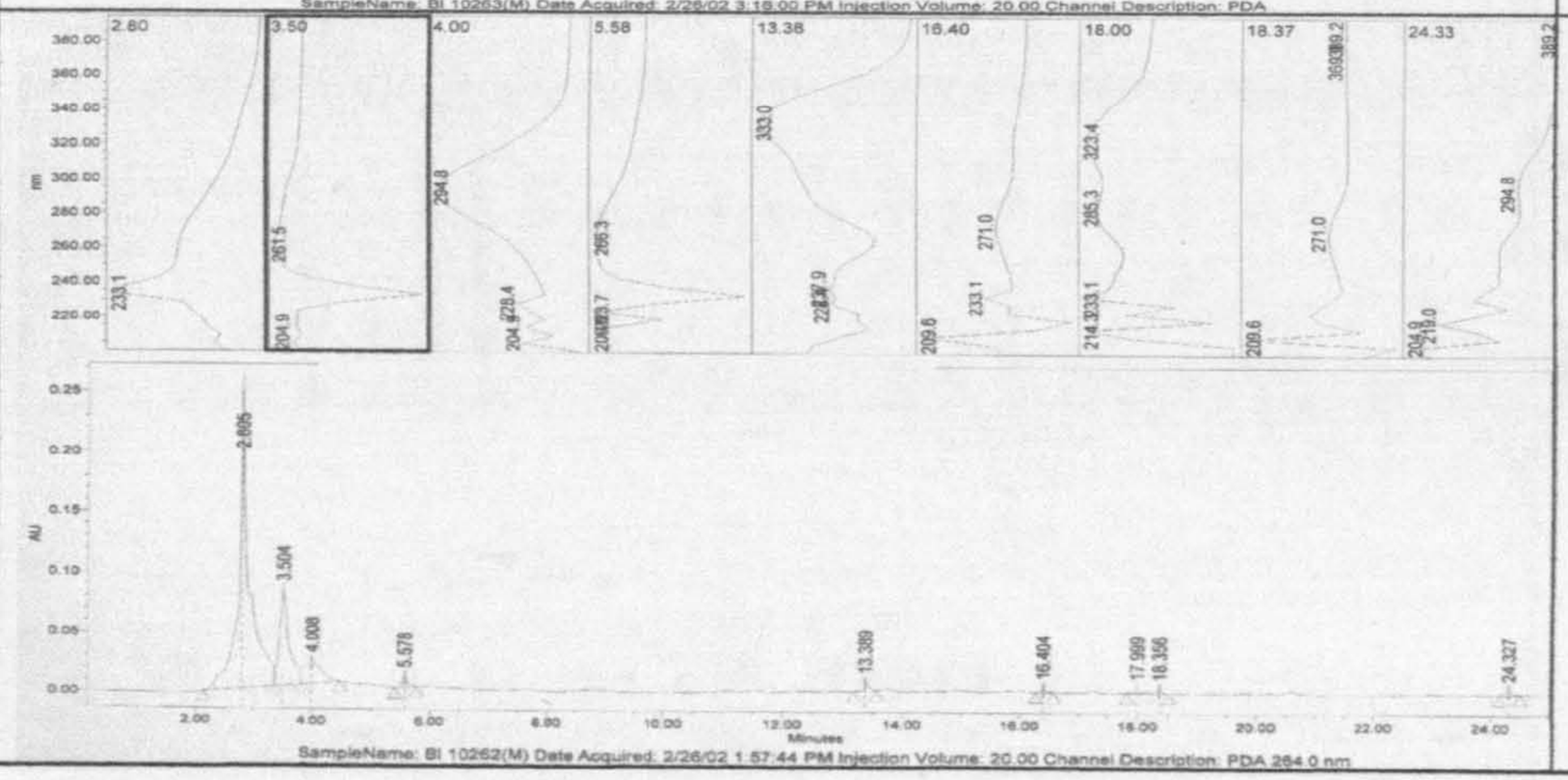
Appendix VIIa cont'd.

TCM names and voucher number	Plant species and family	HPLC fingerprint of samples (MY2) investigated further in chemical and biological assays	Compounds
Huang bai, Phellodendron Cortex (BI 10723)	*Phellodendron amurense* Rupr. (Rutaceae)		Retention time (Rt) 8.76 minutes: caffeic acid derivative Rt 17.15, 17.84 and 18.92 minutes: berberine alkaloids: interesting compounds for markers
Huang lian, Coptidis Rhizoma (BI 10260)	Coptis species Ranunculaceae		SI 9, 10 and 11 minutes: berberine alkaloids:

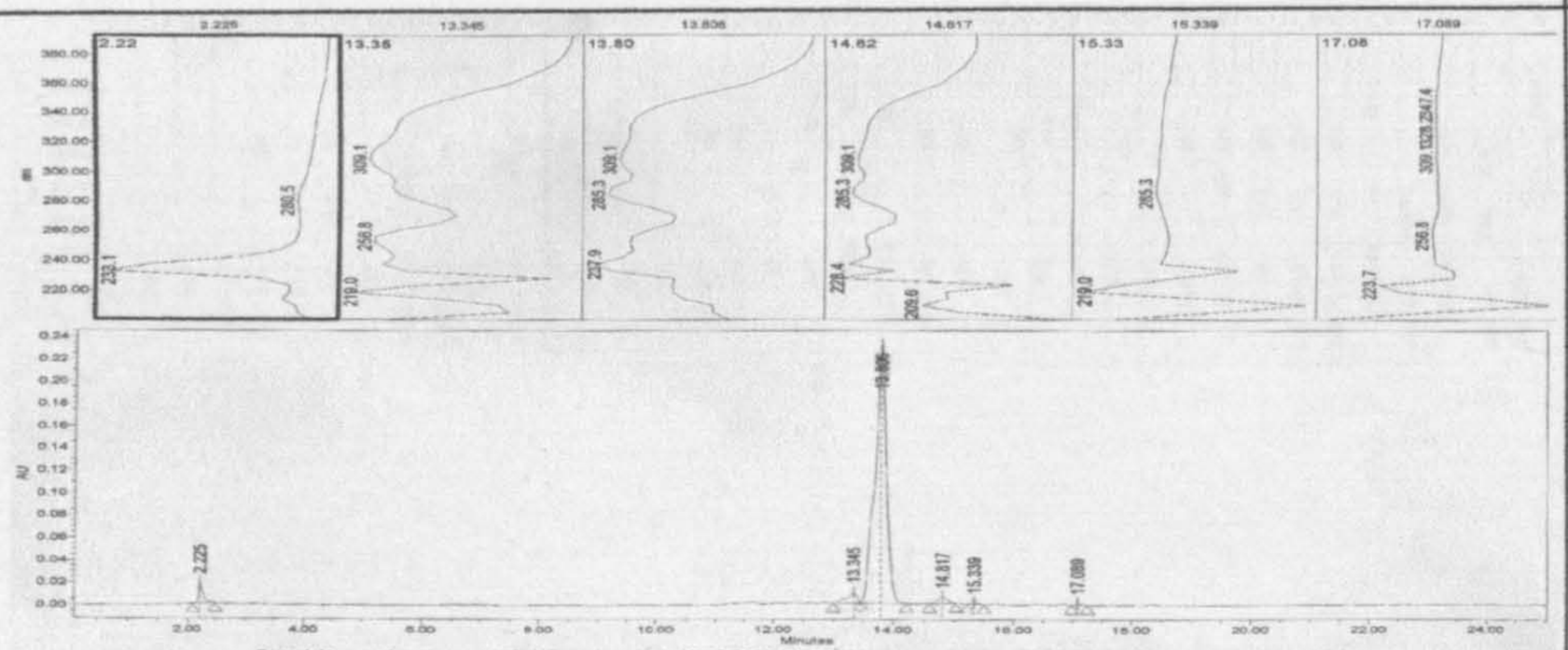
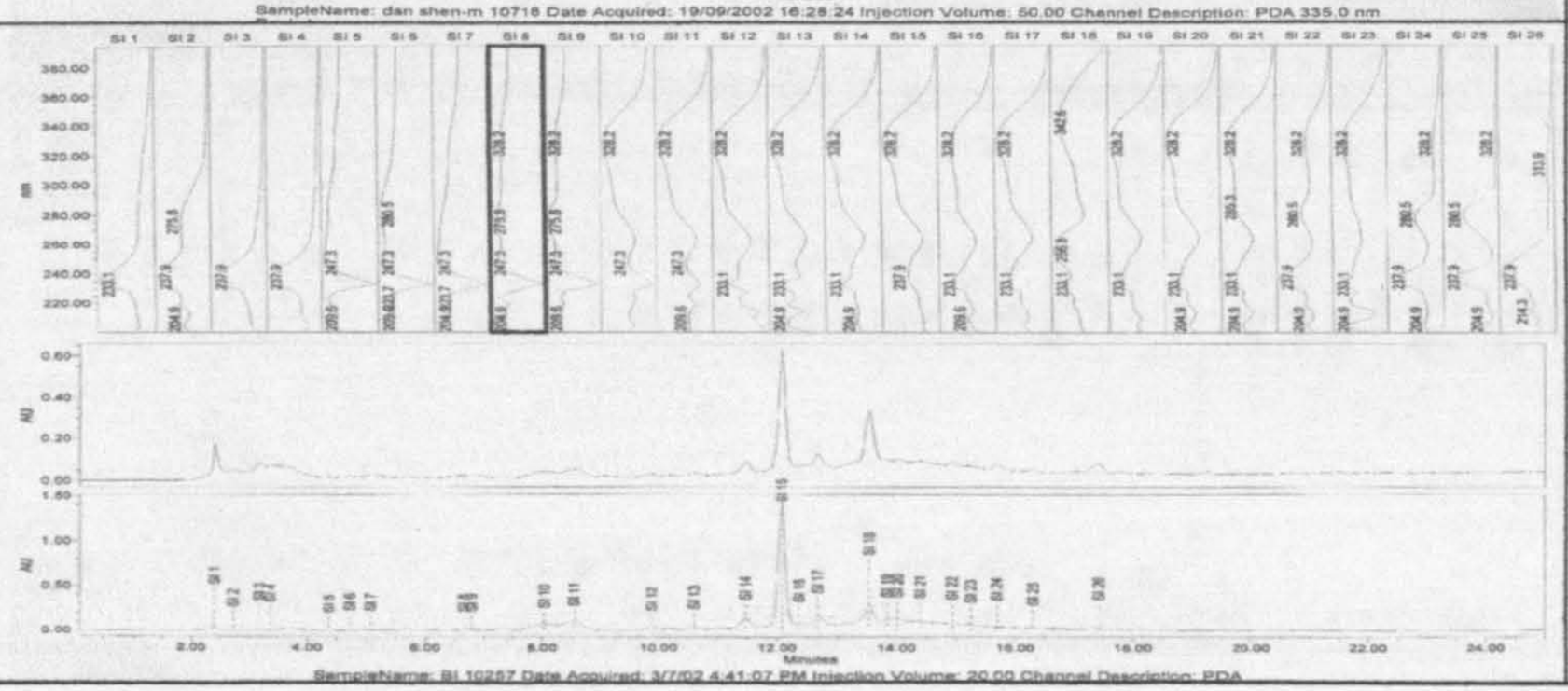
Appendix VIIa cont'd.

TCM names and voucher number	Plant species and family	HPLC fingerprint of samples (MY2) investigated further in chemical and biological assays	Compounds
Da huang, Rhei Rhizoma (BI 10732)	Species not identified. (No available authentic reference material)		None identified

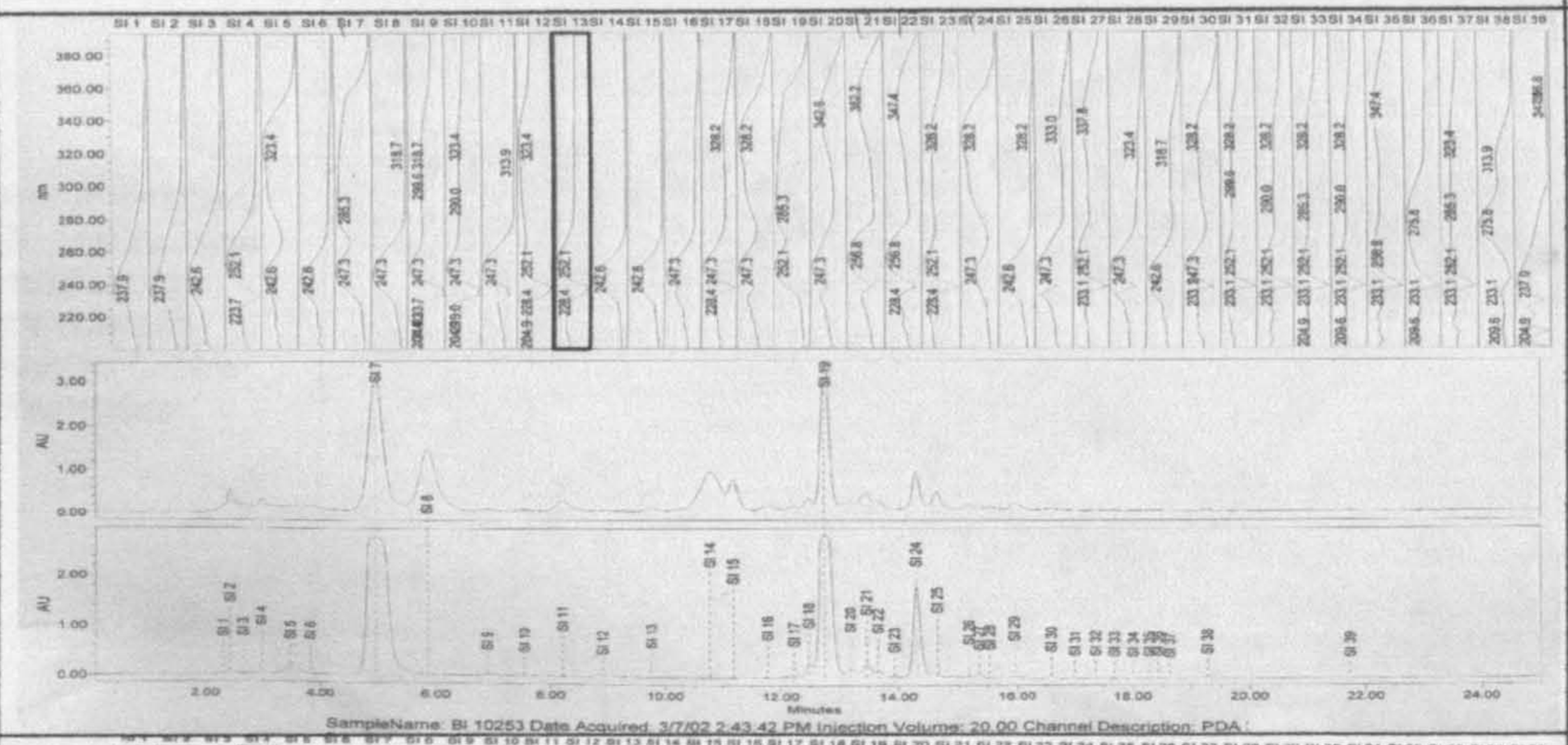
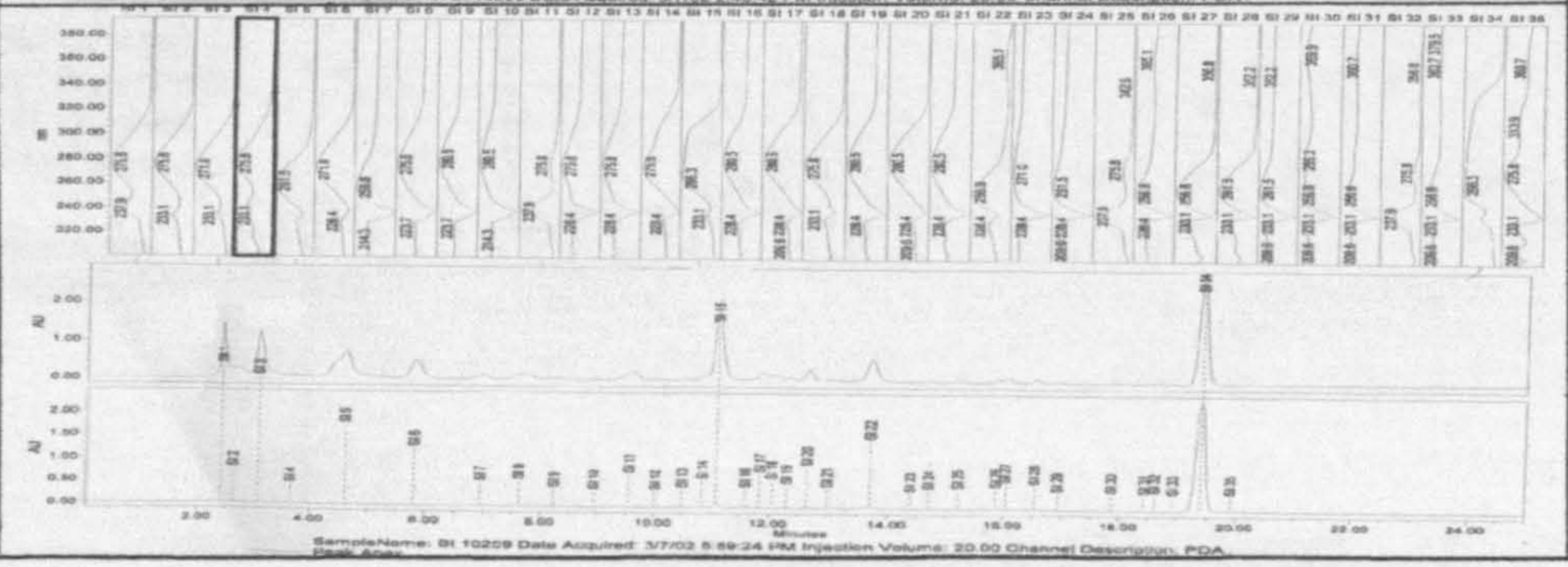
Appendix VIIb. Authentication results for the rhino horn project: information about the Chinese and Latin names of plants used in this study, their voucher numbers and chemical profiles, as well as identified compounds

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Xuan shen, Scrophulariae Radix (BI 10263)	*Scrophularia ningpoensis* Hemsl. (Scrophulariaceae)		SI 2 and 5 - verbascoside-type caffeic acid derivative
Sheng di huang, Rehmanniae Radix (BI 10262)	*Rehmannia glutinosa* Steud. (Scrophulariaceae)		Retention time 13.38 minutes - verbascoside-type caffeic acid derivative

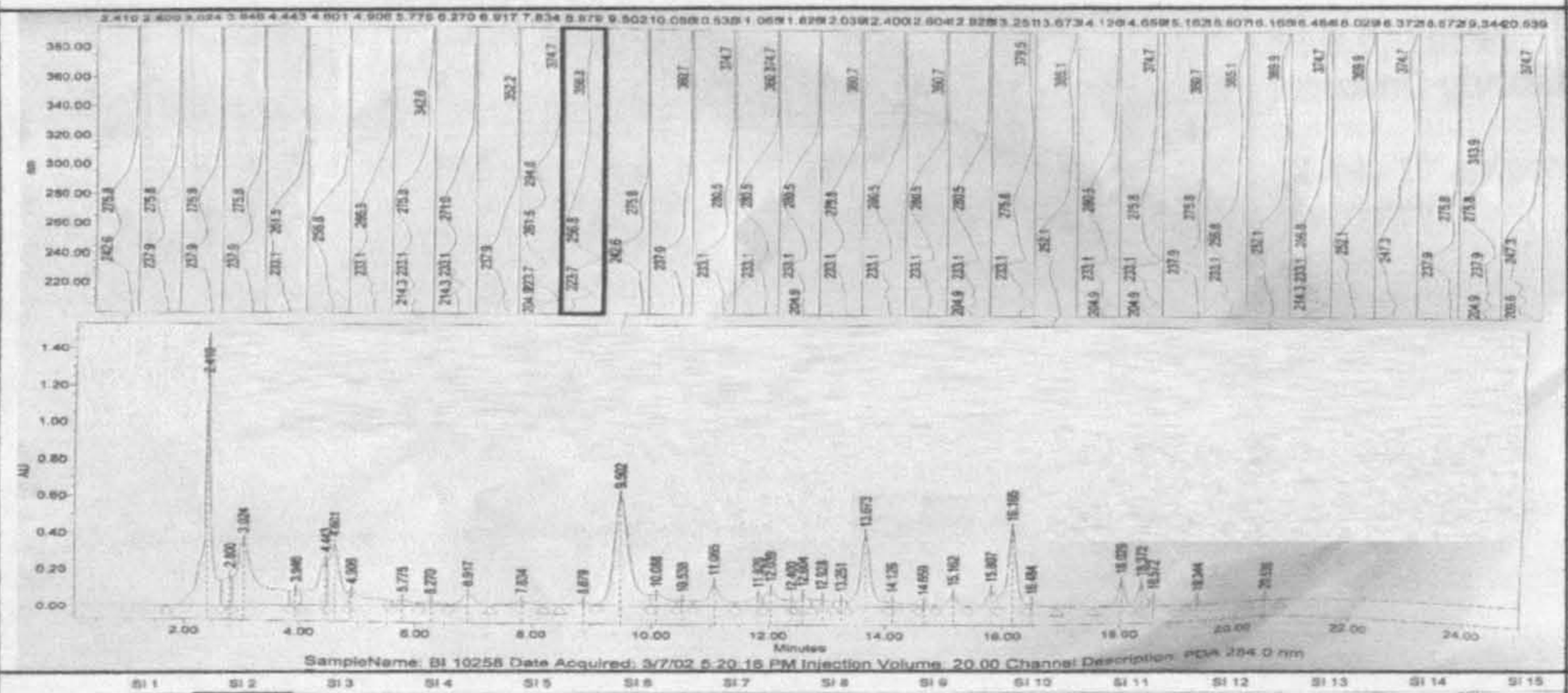
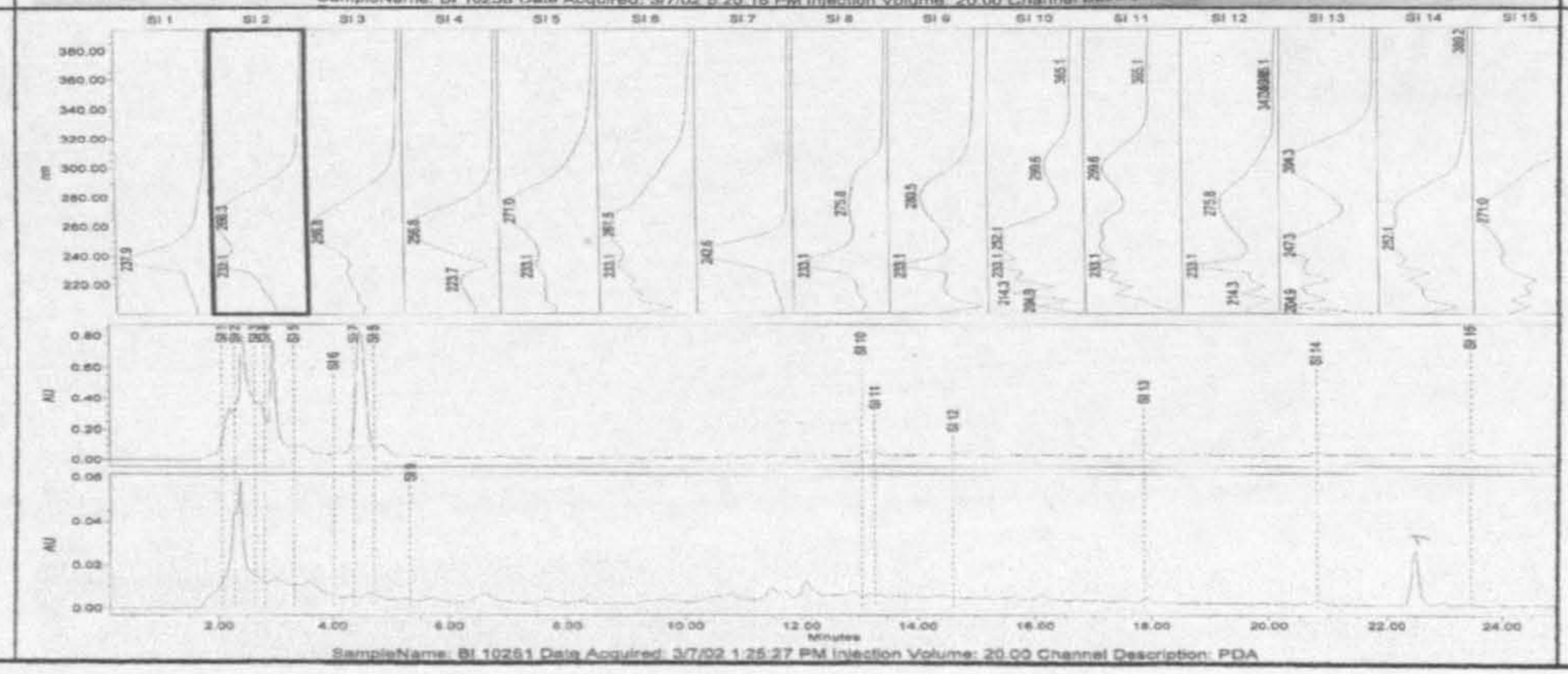
Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Dan shen, Salviae Radix (BI 10718)	*Salvia miltiorrhiza* Bunge (Lamiaceae)		Rt 13.81 rosmarinic acid overloaded and therefore not showing the real UV spectrum of rosmarinic acid
Lian qiao, Forsythia Fructus (BI 10257)	*Forsythia suspensa* Vahl. (Oleaceae)		SI 10, 11, 12, 13, 14, 16, 17, 19, 20 caffeic acid derivatives SI 18 quercetin glycoside

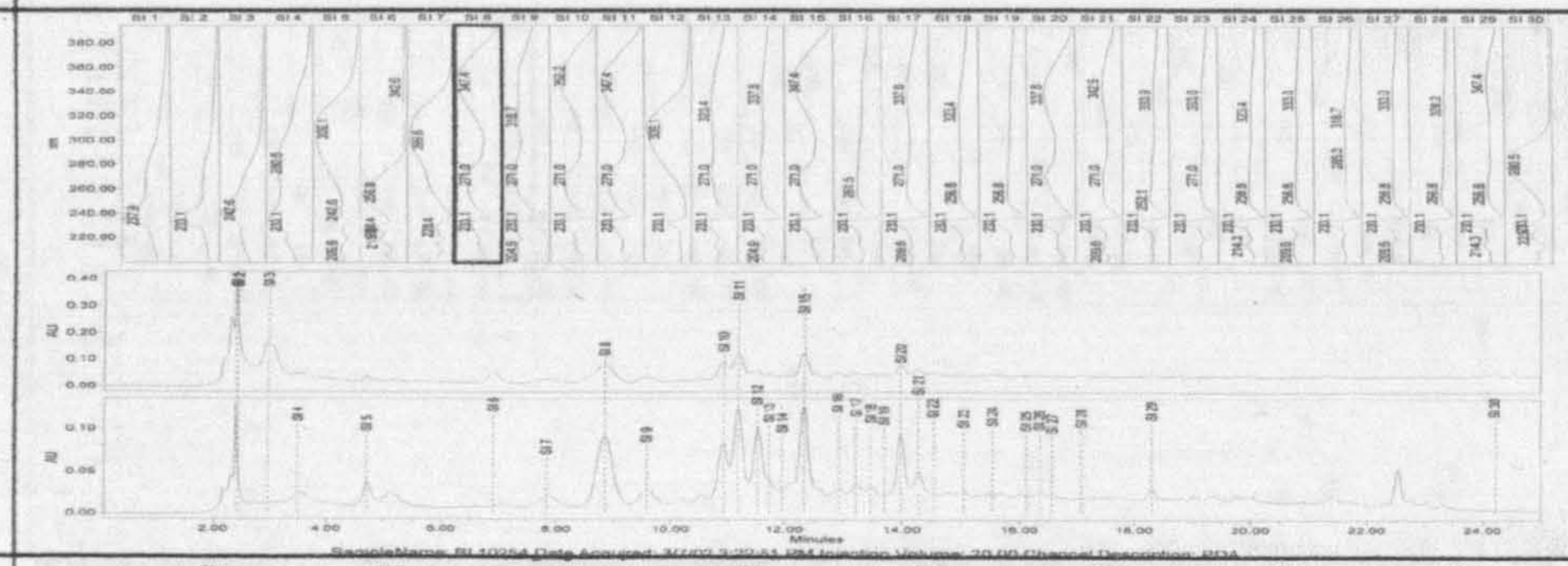
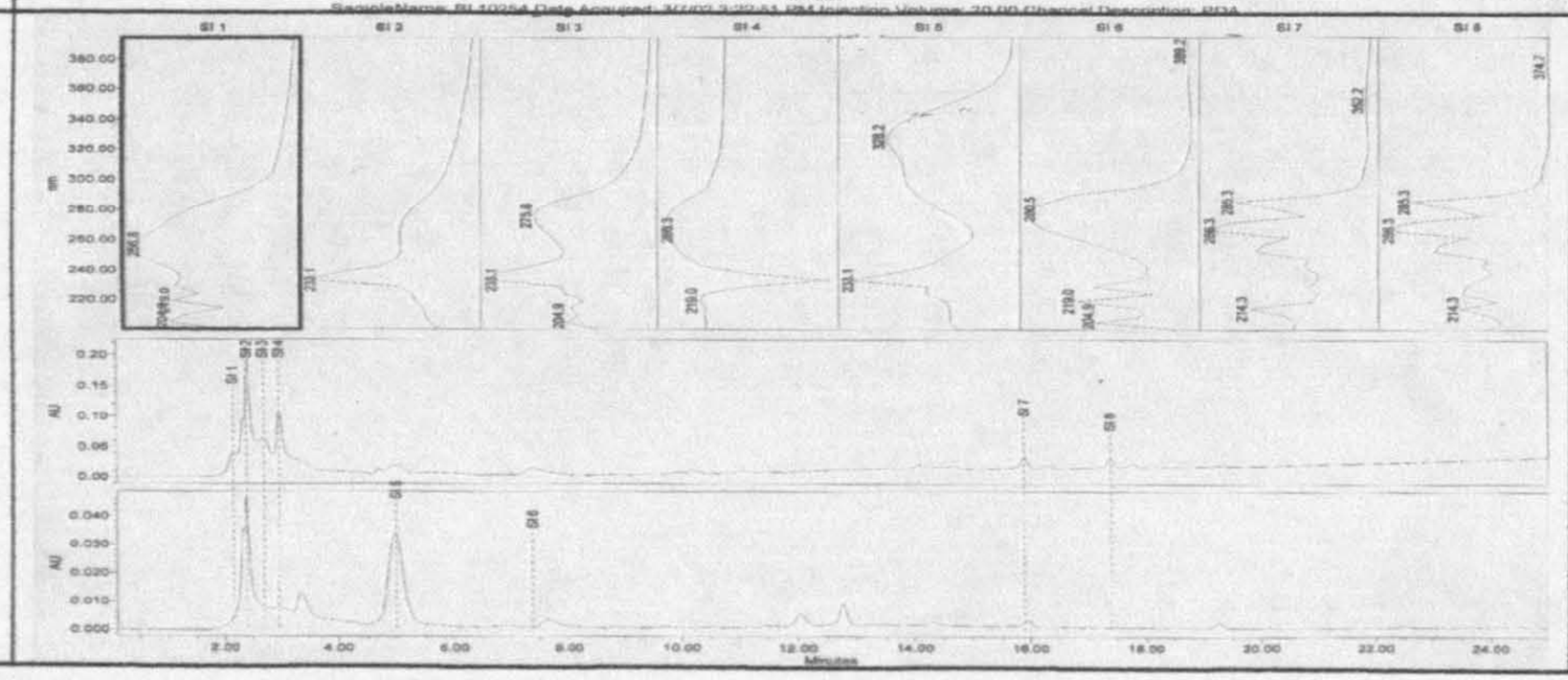
Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Jin yin hua, Lonicerae Herba (BI 10253)	*Lonicera japonica* Thunb. (Caprifoliaceae)		SI 7 and 24: caffeic acid derivatives SI 21 and 22: flavonol glycosides
Mu dan pi, Moutan Radicis Cortex (BI 10259)	*Paeonia suffruticosa* Andr. (Paeoniaceae)		None identified

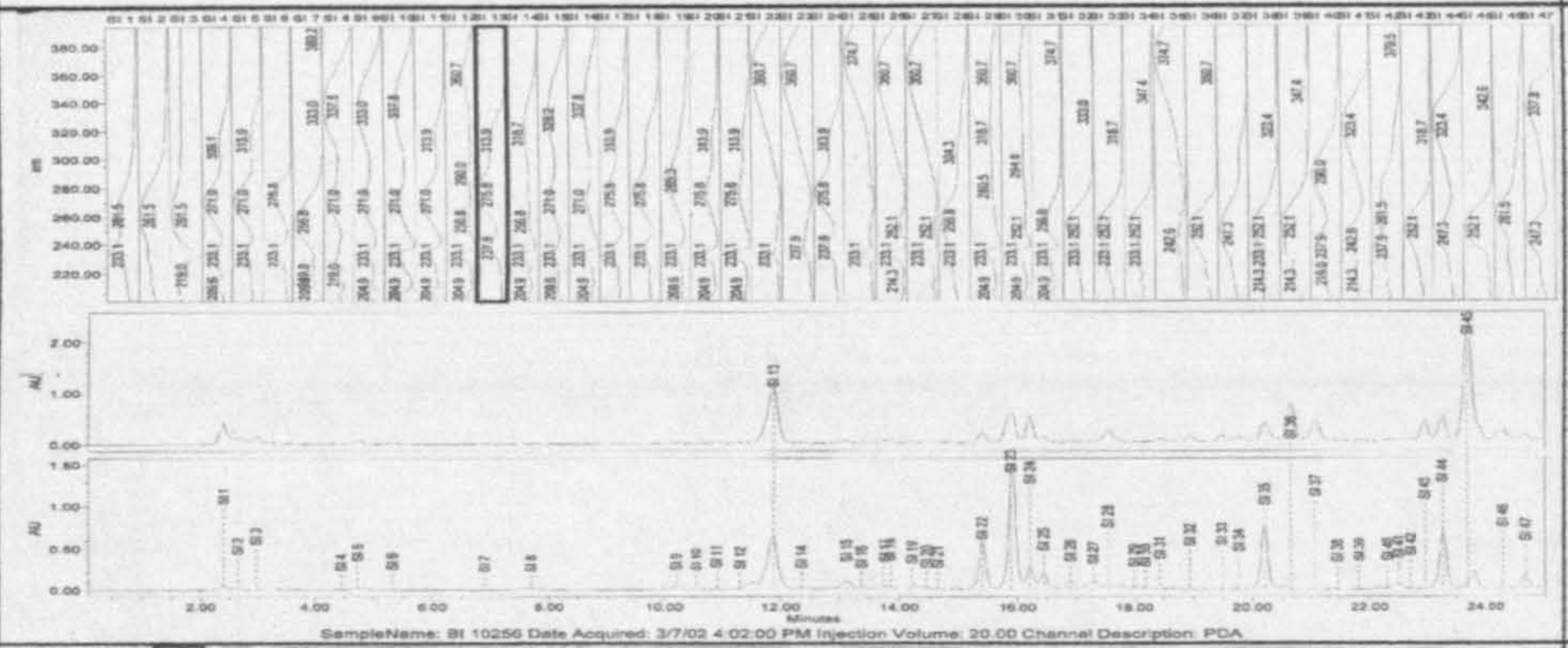
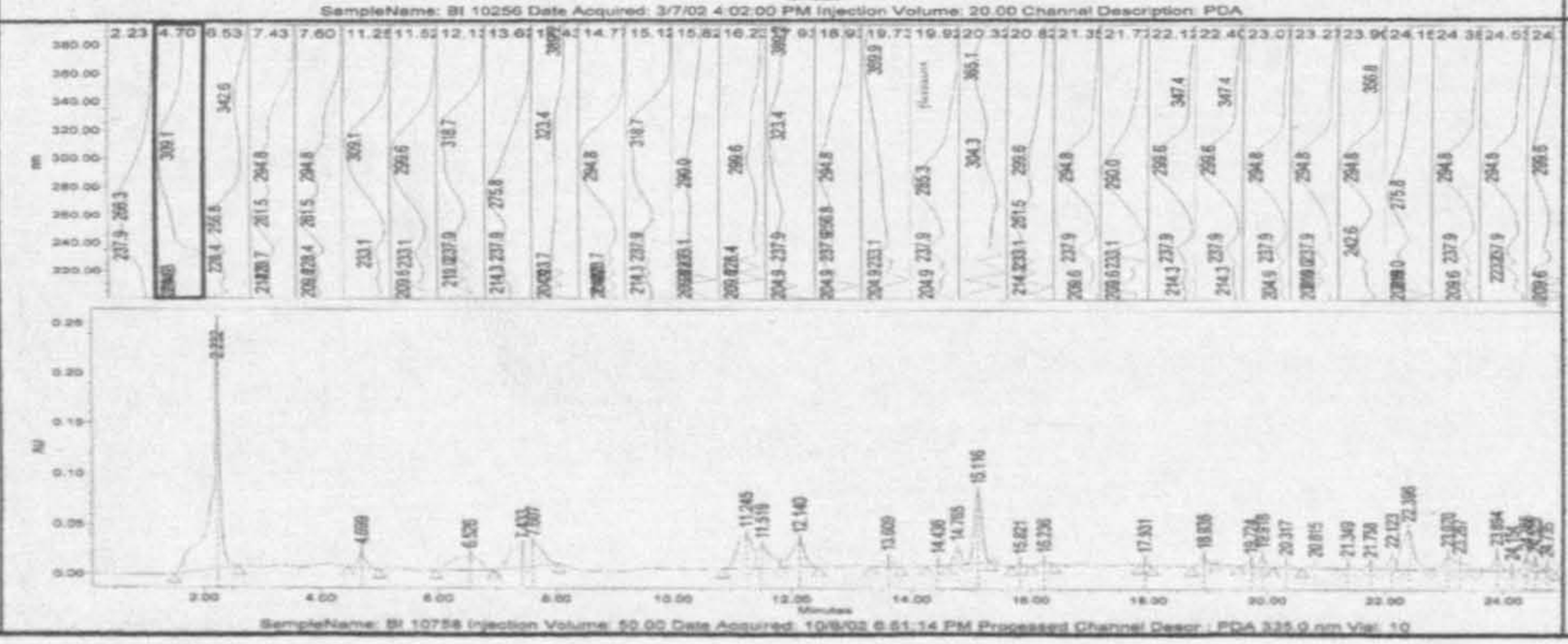
Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Chi shao yao, Paeoniae Rubra Radix (BI 10258)	*Paeonia lactiflora* Pall. and *Paeonia vetchii* Lynch (Paeoniaceae) Requires further investigation		Rt 18.03 and 18.37 minutes ellagic acid derivatives
Ban lan gen, Isatidis Radix (BI 10251)	*Isatis tinctoria* L. (Brasicaceae) or *Baphicacanthus cusia* Bremek (Acanthaceae) Species not identified		None identified

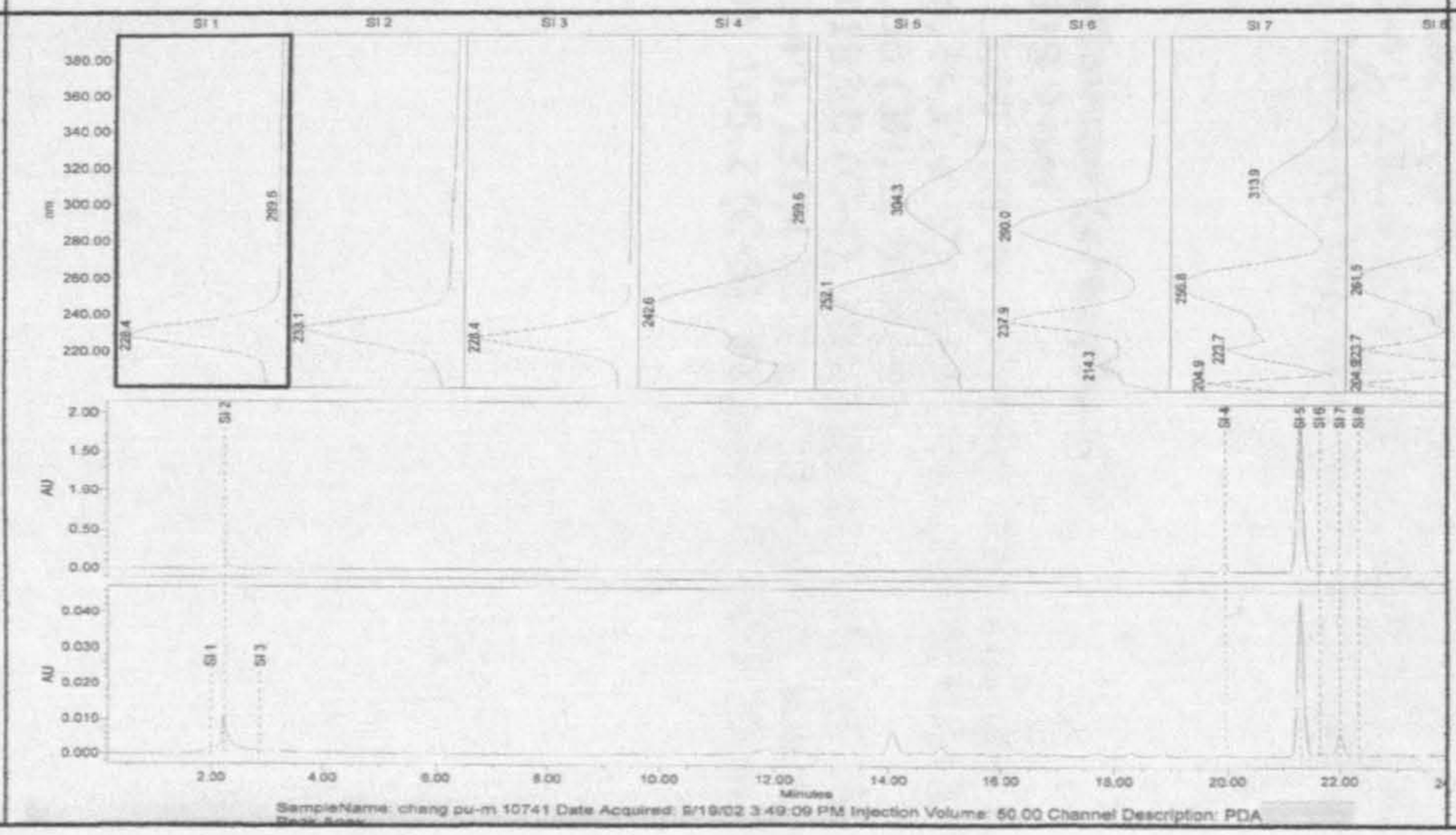
Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Dan zhu ye Lophatheri Herba (BI 10254)	*Lophatherum gracile* Brongn. (Poaceae)		SI 8,9, 10, 11,15 luteolinC-glycosides SI 14, 17 apigenin C-glycosides
Jie geng, Platycodi Radix (BI 10252)	*Platycodon grandiflorum* (Jacq.) A.DC. (Campanulaceae)		SI 5 caffeic acid derivative SI 7 and 8 Polyacetylenes

Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Gan cao Glycyrrhizae Radix (B1 10256)	Profiles indicate sample is a species of *Glycyrrhiza*, and profile is similar to G. *uralensis* Fisch. (Leguminosae,)		SI 12 chalcone SI 13 5-deoxyflavanone SI 8, 10,16 apigenin C-glycoside SI 25, 26, 27, 29, 30, 35 chalcones SI 32, 33, 34, 36, 38, 43, 45 isoflavonoids
Mai men dong Ophiopogonis Radix (BI 10758)	*Ophiopogon* species. (Liliaceae)		Rt 19.92 minutes flavanone

Appendix VIIb cont'd.

TCM names	Plant species, Plant family & comments	HPLC profile of samples (MY2) investigated further in chemical and biological assays	Compounds
Lian zi xin, Nelumbinis Nucifera Plumula (BI 10757)	*Nelumbo nucifera* Gaertn., Nelumbonaceae,		
Chang pu, Acori Graminei Rhizoma (B1 10741)	*Acorus gramineus*[Soland.], (Araceae)		None identified

Appendix VIII. Data for the characterisation of wogonin and oroxylin A

5,7-Dihydroxy-8-methoxyflavone (wogonin):
UV (MeOH) λmax = 275.8 nm
^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.48 (1H, s, 5-OH), 7.91 (2H, dd, J = 7.6, 1.5 Hz, H-2',6'), 7.56 (3H, m, H-3',4',5'), 6.68 (1H, s, H-3), 6.49 (1H, s, H-6), 6.32 (1H, br s, 7-OH), 4.04 (3H, s, OCH_3-8);
^{13}C NMR ($CDCl_3$, 100 MHz) δ 182.4 (C-4), 163.5 (C-2), 157.9 (C-5), 155.4 (C-7), 148.8 (C-9), 131.9 (C-4'), 131.4 (C-1'), 129.1 (C-3',5'), 126.9 (C-8), 126.1 (C-2',6'), 105.8 (C-3), 105.4 (C-10), 98.8 (C-6), 61.9 (OCH_3-8).

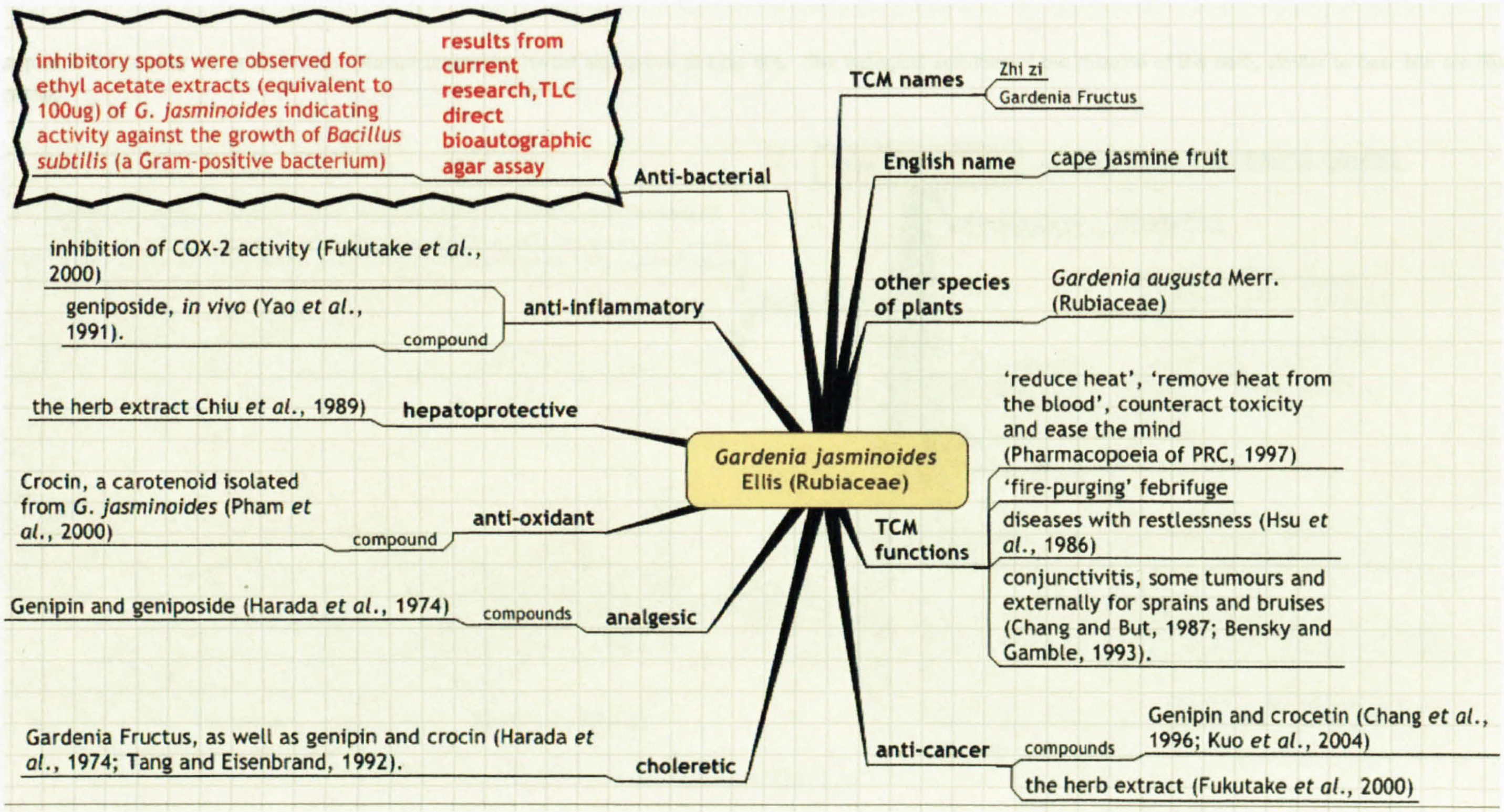
5,7-Dihydroxy-6-methoxyflavone (oroxylin A):
UV (MeOH) λmax = 271.0, 318.7 nm
^{1}H NMR ($CDCl_3$, 400 MHz) δ 13.00 (1H, br s, 5-OH), 7.88 (2H, dd, J = 7.6, 1.3 Hz, H-2',6'), 7.55 (3H, m, H-3',4',5'), 6.66 (1H, s, H-3), 6.61 (1H, s, H-8), 6.50 (1H, br s, 7-OH), 4.06 (3H, s, OCH_3-6);
^{13}C NMR ($CDCl_3$, 100 MHz) δ 183.0 (C-4), 164.2 (C-2), 155.1 (C-7), 154.4 (C-5), 153.4 (C-9), 131.6 (C-4'), 131.4 (C-1'), 130.4 (C-6), 128.9 (C-3',5'), 126.2 (C-2',6'), 106.0 (C-10), 105.2 (C-3), 93.2 (C-8), 60.7 (OCH_3-6).

Appendix IX. Summary of anti-bacterial and anti-inflammatory results for 24 TCM herbs

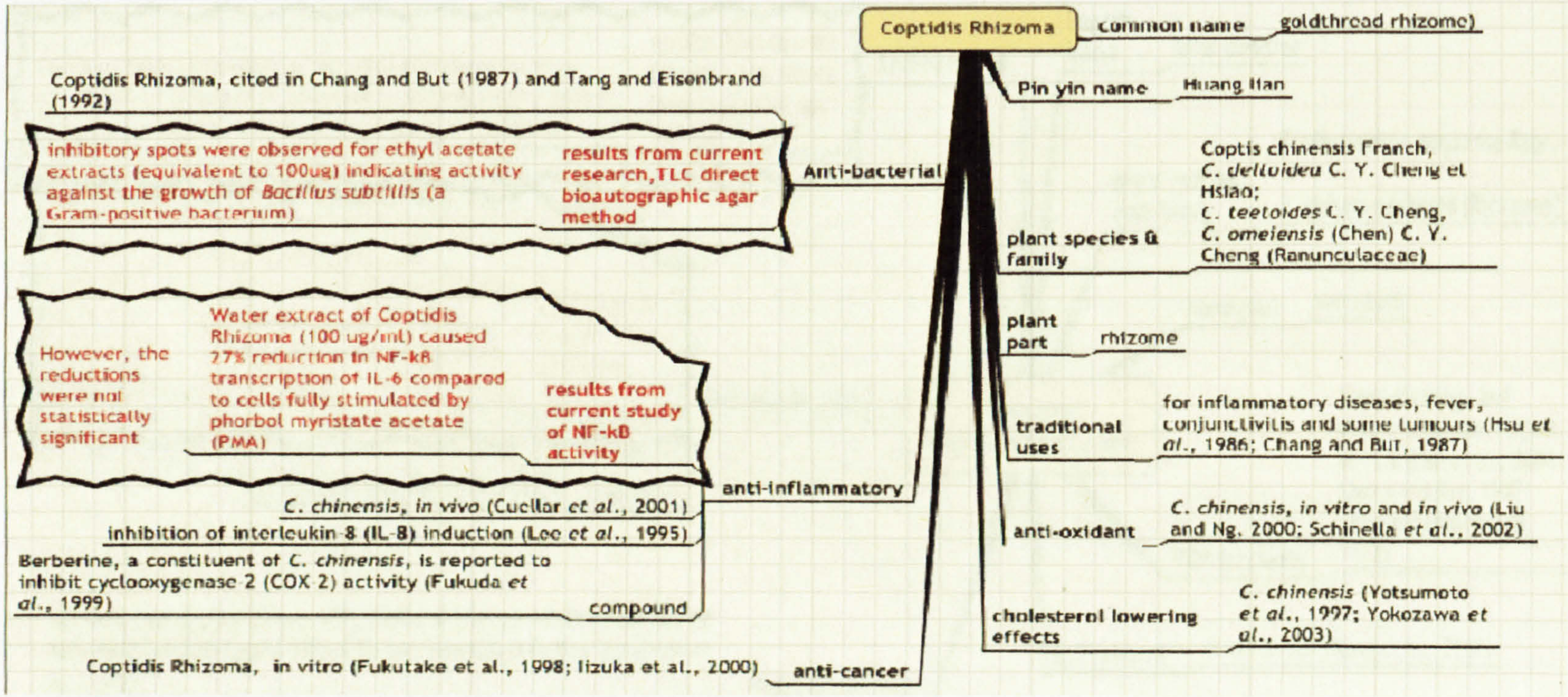
Samples (the names of plant species confirmed in this study are given in italics)	Anti-bacterial (*Bacillus subtilis*) tests ethyl acetate extracts, 100 µg	Anti-inflammatory tests (inhibition NF-κB activity, unless otherwise stated), 100 µg/ml	
1. Rhei Radix et Rhizoma (da huang, DH)	+	W: 18%, p<0.05	Fig. 6.3
2. *Phellodendron amurense* (huang bai, HB)	+	W, 35%	Fig. 6.3
3. *Scutellaria baicalensis* (huang qin, SB)	+	F: inhibition, p<0.01 E: 69%, p<0.01	Figs. 6.3, 6.4, 6.5
4. *Anemarrhena asphodeloides* (zhi mu, ZM)	+	W:8%	Fig. 6.3
5. *Gardenia jasminoides* Ellis (zhi zi, ZZ)	+	nt	
6. Coptidis Rhizoma (huang lian; HL)	+	W: 27%	Fig. 6.3
7. *Andrographis paniculata* (chuan xin lian, CXL)	-	W: 8%, stimulation	Fig. 6.3
8. Trichosanthis Radix (tian hua fen, THF)	-	W: 50%, p<0.05	Fig. 6.2
9. Isatidis Radix (ban lan gen, BLG)	+	W: 33%, p<0.05	Fig. 6.2
10. *Salvia miltiorrhiza* Bge. (dan shen, SM)	+	F: (SM6, 98.9%), (SM7, 98%), (SM8, 52%)	Fig. 6.7
11. *Lophatherum gracile* (dan zhu ye, DZY)	+	W: 46%, p<0.05	Fig. 6.2
12. *Lonicera japonica* (jin yin hua, JYH)	+	W: 2%	Fig. 6.2
13. *Forsythia suspensa* Vahl (lian qiao, LQ)	+	nt	
14. *Paeonia suffruticosa* (mu dan pi, MDP)	nt	W: 56%, p<0.001	Fig. 6.2
15. *Rehmannia glutinosa* (sheng di huang, SDH)	+	W: 14%, p<0.05	Fig. 6.2
16. *Scrophularia ningpoensis* (xuan shen, XS)	+	W: 6%	Fig. 6.2
17. Arnebiae seu Lithospermi Radix (zi cao, ZC)	+	nt	
18. Sojae Praeparatum Semen (dan dou chi, DDC)	+	W: 18%, p<0.05	Fig. 6.2
19. *Platycodon grandiflorum* (jie geng, JG)	+	W: 3%	Fig. 6.2
20. Ophiopogonis Radix (mai men dong, MMD)	+	nt	
21. Acori Graminei Rhizoma (chang pu, CP)	-	W: 38%	Fig. 6.2
22. *Paeonia lactiflora* (chi shao yao, CSY)	-	W: 34%	Fig. 6.2
23. *Glycyrrhiza uralensis* (gan cao, GC)	-	W: 18%stimulation	Fig. 6.2
24. Nelumbinis Semen (lian zi xin, LZX)	-	nt	

+ = inhibition (activity), - = no inhibition (no activity), nt = not tested, F = fractions, E = ethyl acetate extract, W = water extract. Samples 1 to 8 are proposed as herbal alternatives to bear bile and samples 9 to 17 as alternatives to rhino horn.

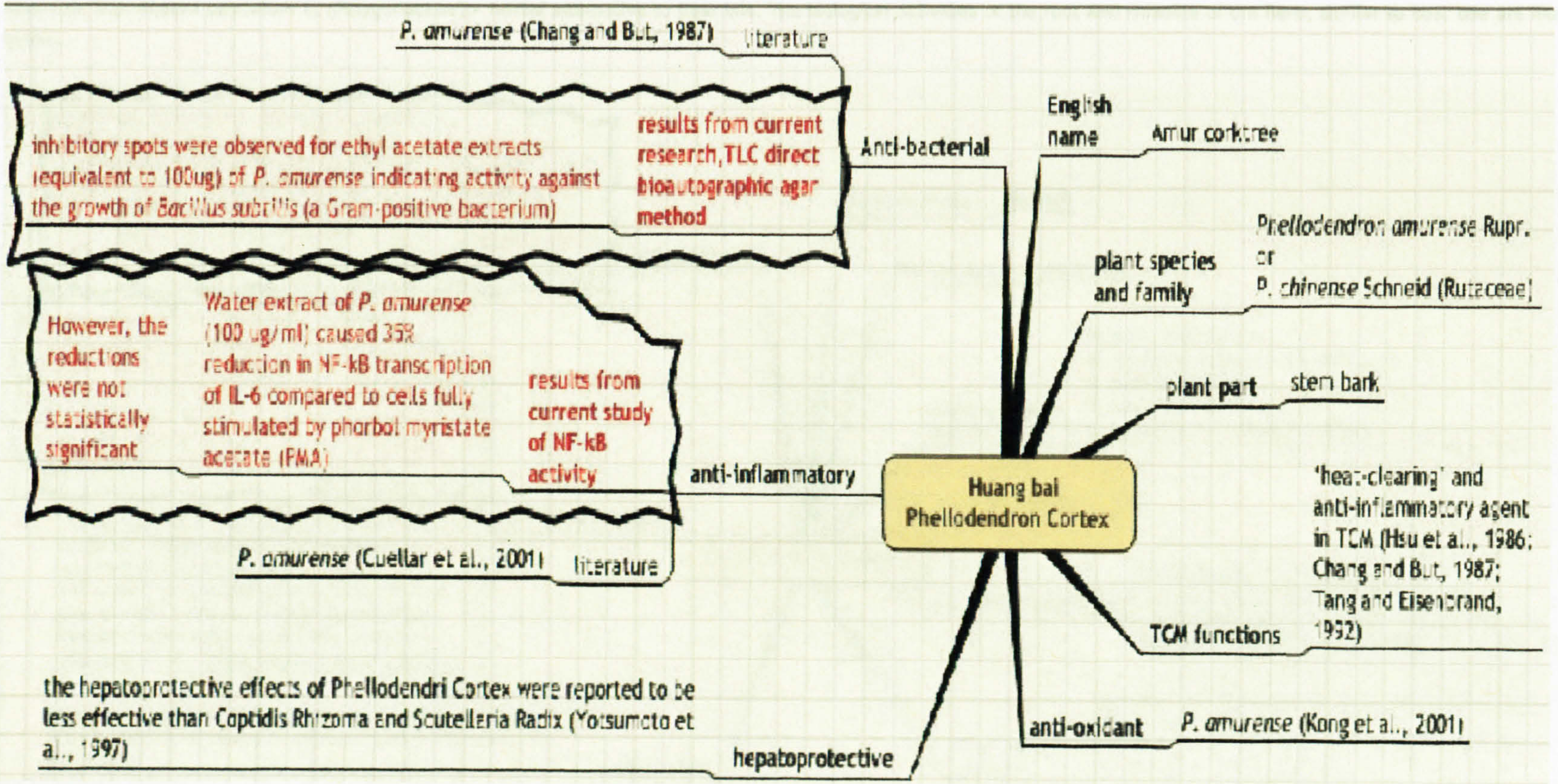
Appendix IXa. *Gardenia jasminoides* Ellis (Rubiaceae):- herbal alternative to bear bile. The biological activities of the fuit of the herb, similar to bear bile is illustrated below



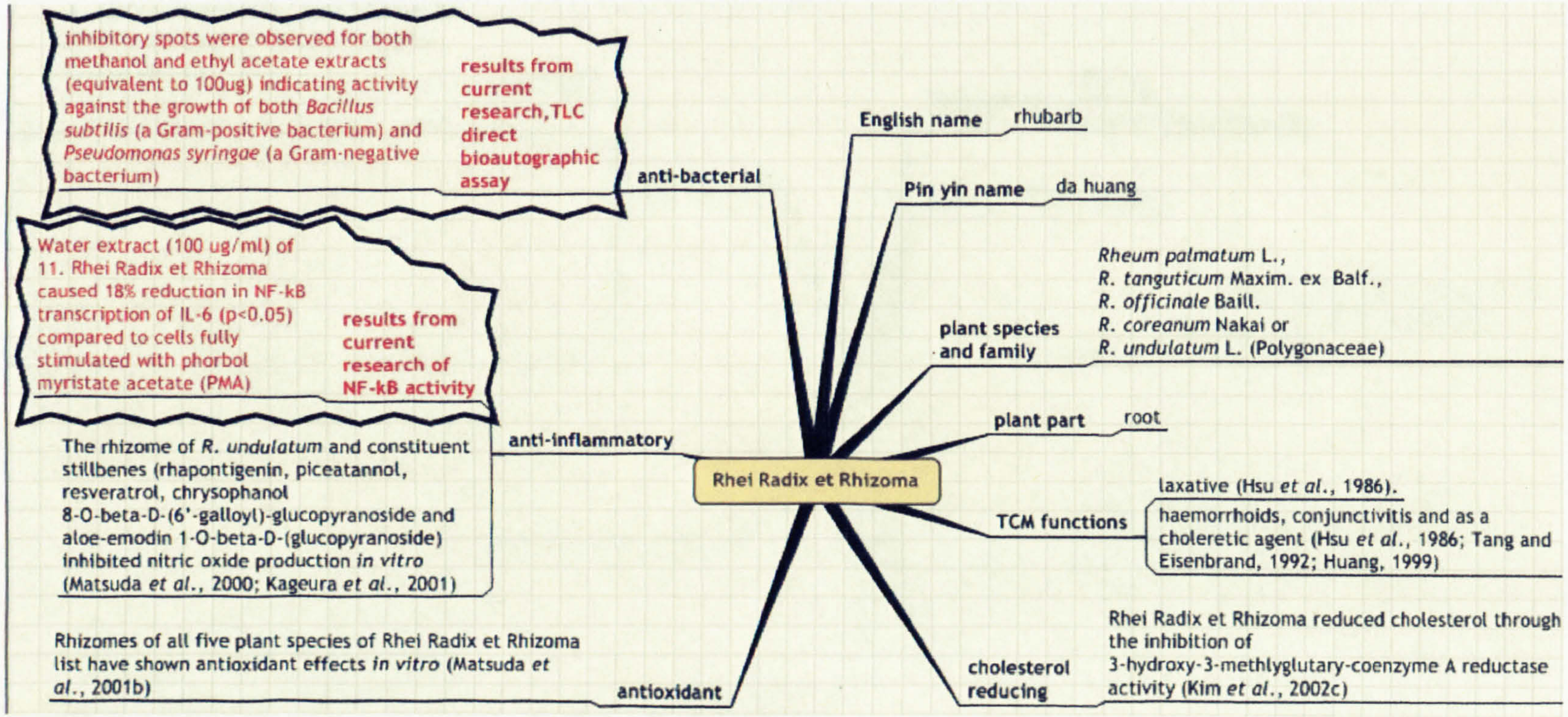
Appendix IXb. *Coptis chinensis* Franch. (Ranunculaceae):- herbal alternative to bear bile. The biological activities of the rhizome of the herb, similar to bear bile are illustrated below.



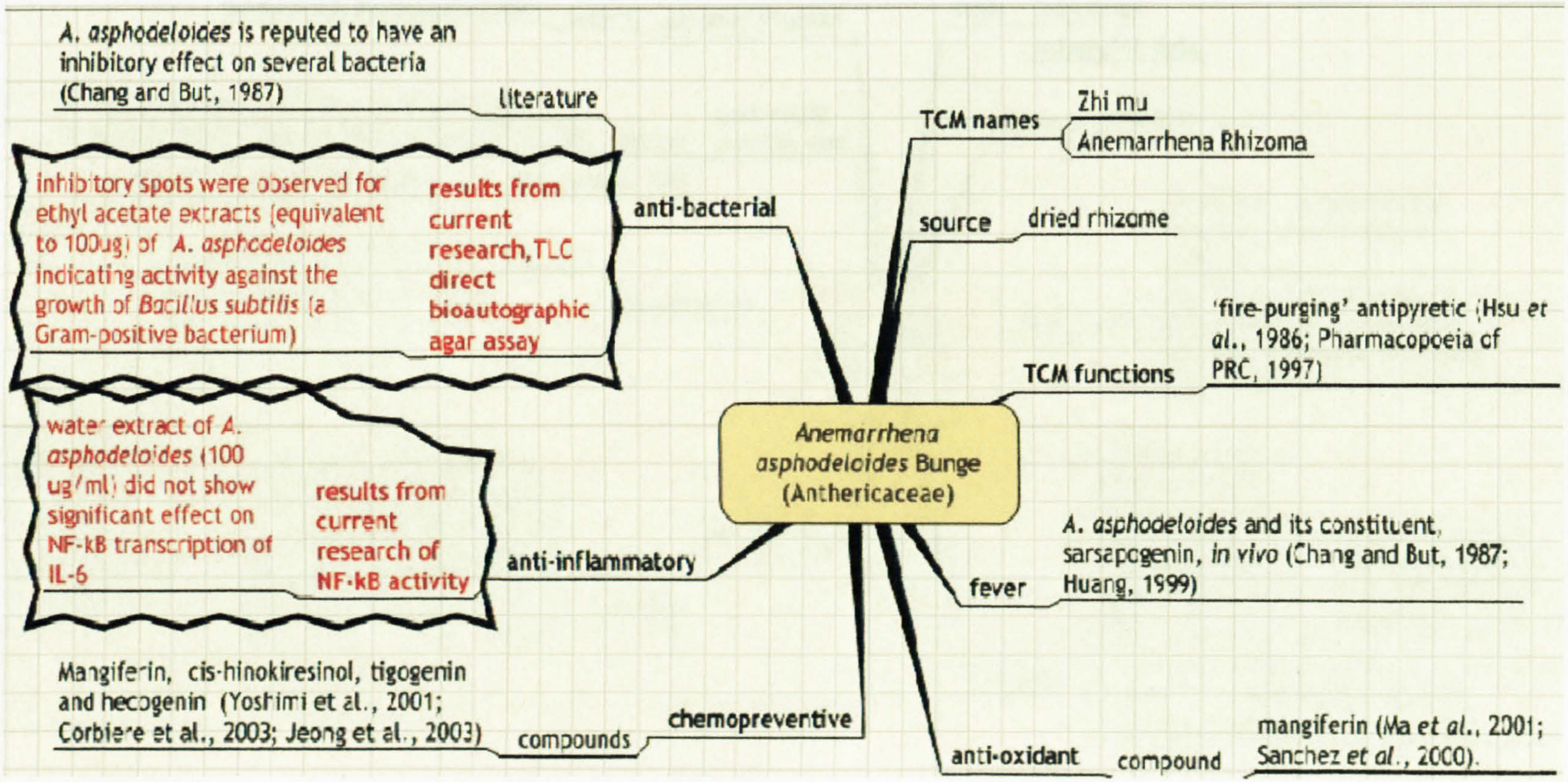
Appendix IXc. *Phellodendron amurense* Rupr. or *P. chinense* Schneid. (Rutaceae):- herbal alternative to bear bile. The biological activities of the stem bark of the herb, similar to bear bile are illustrated below.



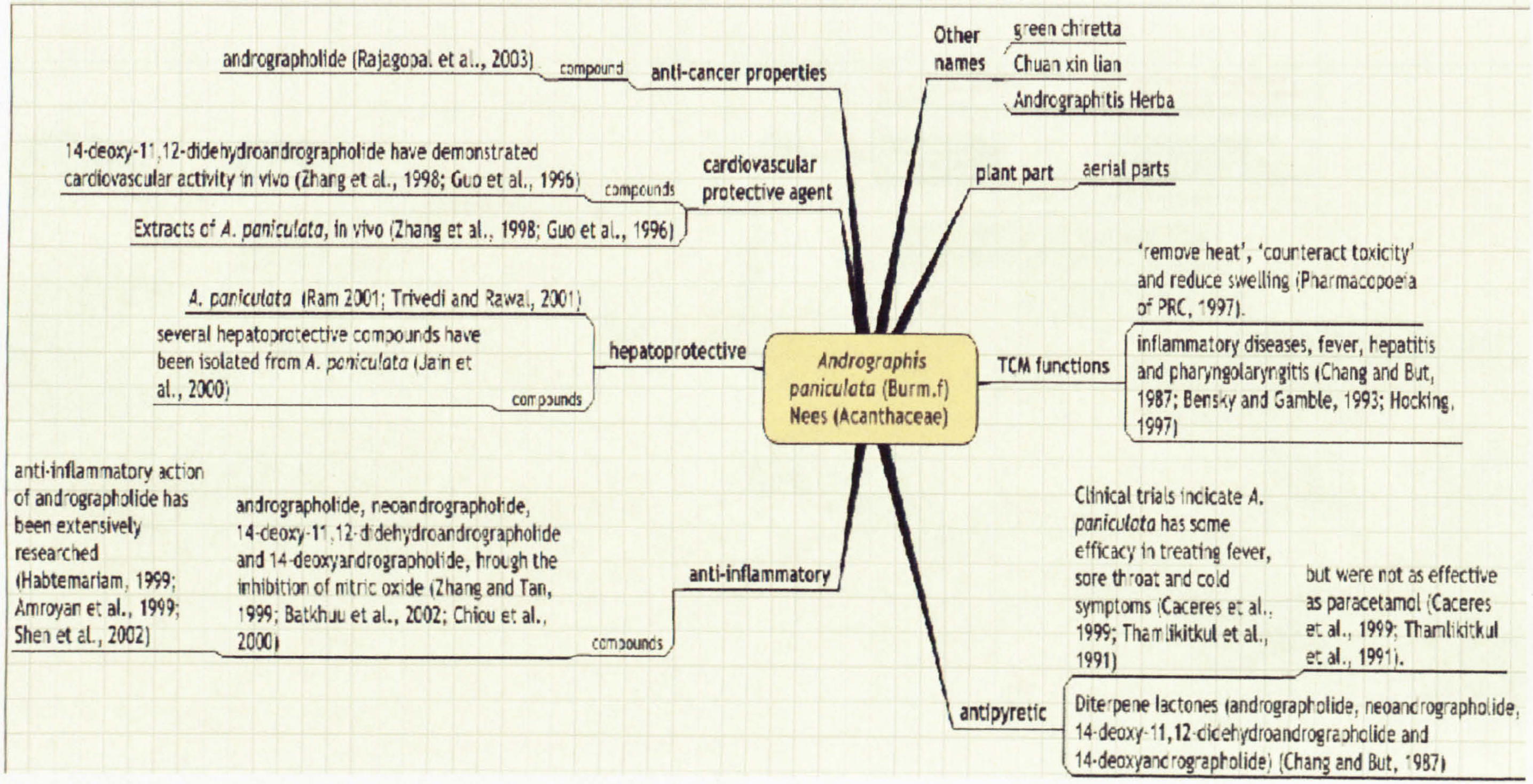
Appendix IXd. *Rheum palmatum* L. (Polygonaceae):- herbal alternative to bear bile. The biological activities of the root and rhizome of the herb, similar to bear bile are illustrated below.



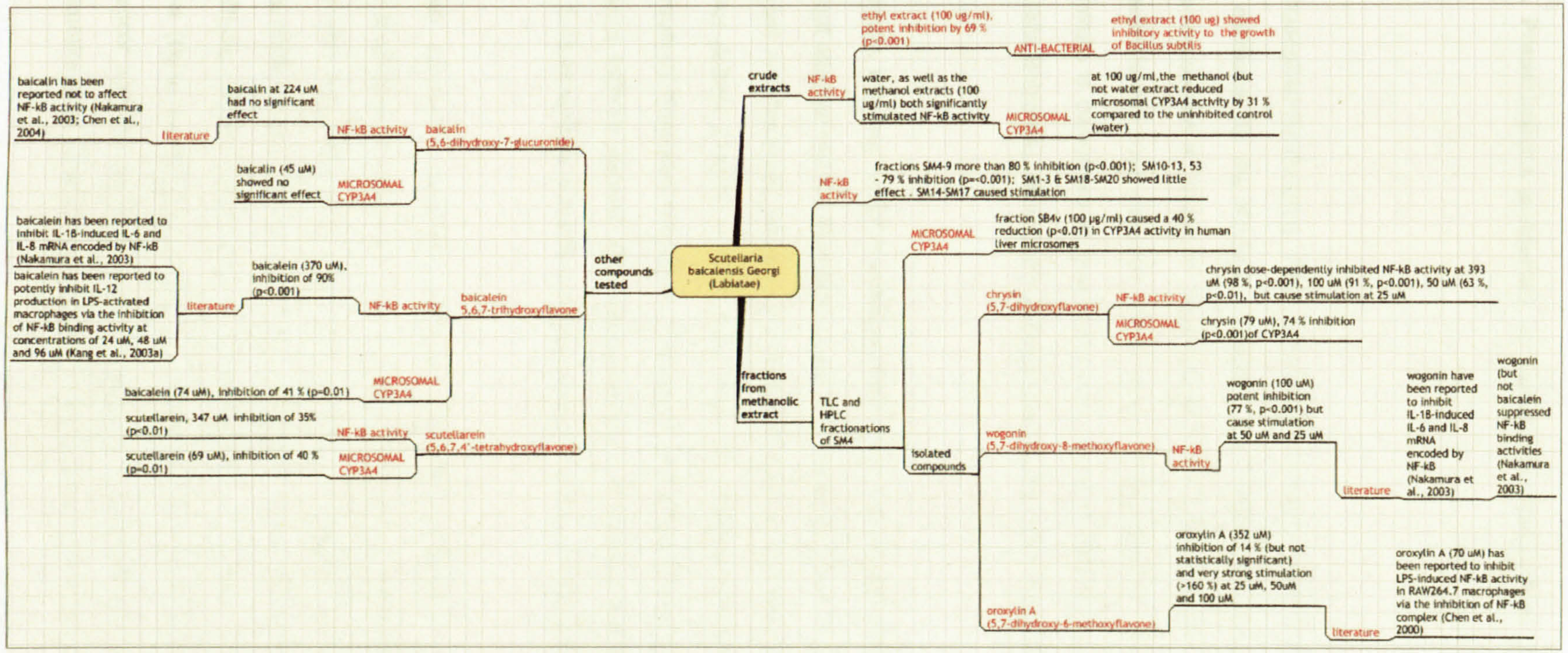
Appendix IXe. *Anemarrhena asphodeloides* Bge. (Anthericaceae):- herbal alternative to bear bile. The biological activities of the rhizome of the herb, similar to bear bile are illustrated below.



Appendix IXf. *Andrographis paniculata* Nees (Acanthaceae):- herbal alternative to bear bile. The biological activities of the aerial part of the herb, similar to bear bile are illustrated below.



Appendix IXg. *Scutellaria baicalensis* Georgi (Labiatae):- herbal alternative to bear bile. The biological activities of the root of the herb, similar to bear bile are illustrated below.



Appendix IXh. Prescriptions proposed as potential alternatives for bear bile

Orengedokuto

Orengedokuto (huanglian-jie-du-tang, TJ-15) is a traditional kampo medicine which is an approved medicine by the Ministry of Health and Welfare of Japan, and is listed in the Pharmacopoeia of Japan for the treatment of cerebrovascular disease, hypertension, gastritis and liver diseases (Ohta et al., 1998; Maclean and Taylor, 2000). Orengedokuto is composed of four herbs, huang bai (Phellodendri Cortex), huang lian (Coptidis Rhizoma), huang qin (Scutellariae Radix) and zhi zi (Gardenia Fructus)

In this current study, the four herbs in orengedokuto were individually studied and proposed as herbal alternatives for bear bile. Crude ethyl acetate extracts of all four herbs demonstated anti-bacterial activity against the growth of the Gram-positive bacterium *Bacillus subtilis* (Tables 5.3) in this study. In addition, some fractions obtained from aqueous methanol extract of *Scutellaria baicalensis* (huang qin) was also had inhibitory activity against the Gram-negative bacterium, *Pseudomonas syringae* (Table 5.4) as well as anti-inflammatory activities (Fig. 6.4) in the present study.

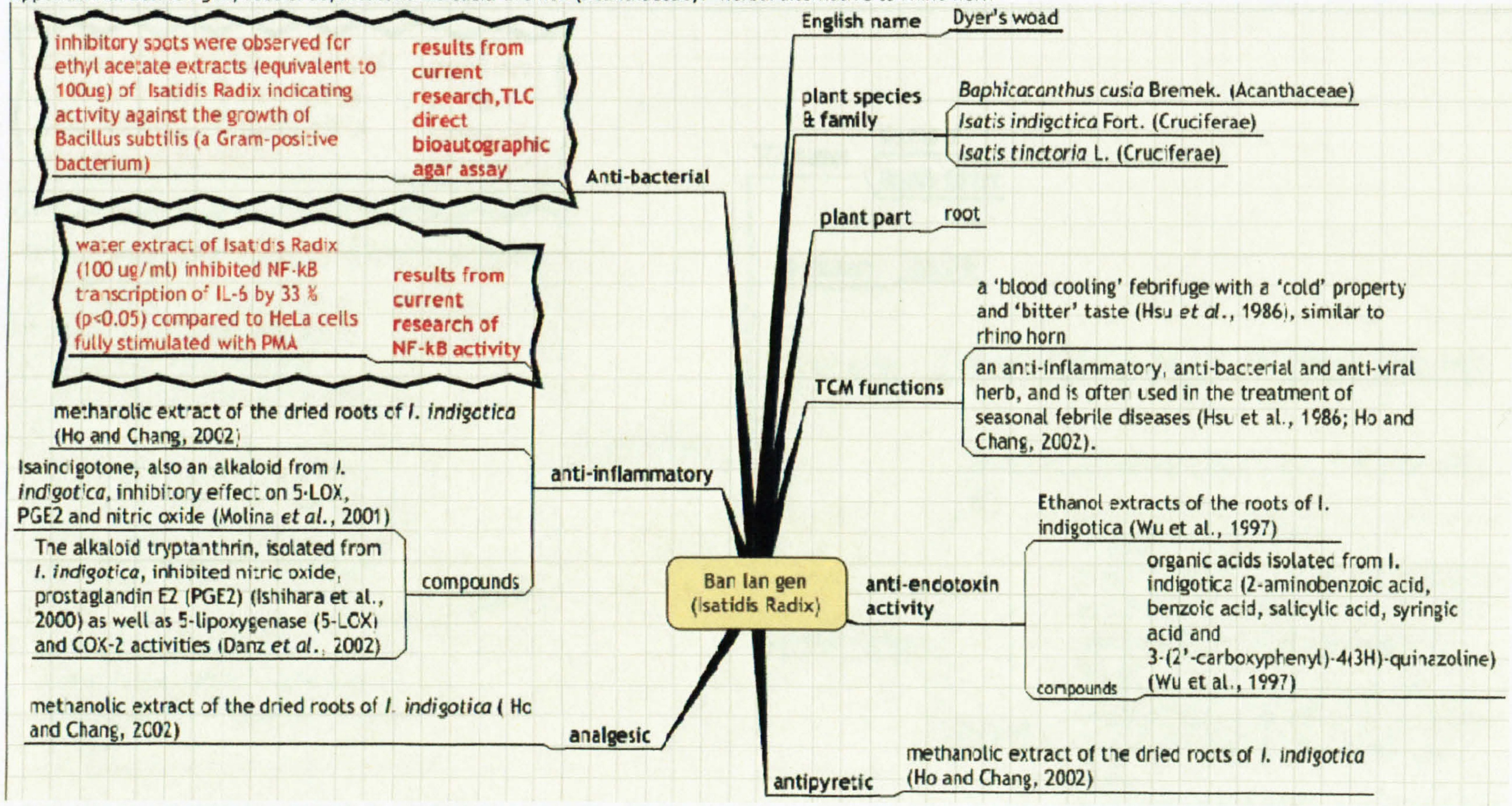
There has been extensive scientific research, by other workers, into the pharmacological properties of orengedokuto. Similar to bear bile, orengedokuto has been associated with anti-inflammatory (Dai *et al.*, 2000; Fukutake *et al.*, 2000), hepatoprotective (Ohta *et al.*, 1998; Sekiya *et al.*, 2002) and anti-cancer (Fukutake *et al.*, 2000) effects. Water extracts of Coptidis Rhizoma, Scutellaria Radix and Gardenia Fructus (three of the components of orengedokuto) also showed some potential anti-cancer activity, but with lower efficacy than orengedokuto (Fukutake *et al.*, 1998; Fukutake *et al.*, 2000). Orengedokuto has also been reported to inhibit

hepatic cholesterol ester formation by inhibiting the activity of acyl-coenzymeA:cholesterol acyltransferase (ACAT) *in vitro* (Yotsumoto *et al.*, 1997). In addition, extracts of Scutellaria Radix, Coptidis Rhizoma and Phellodendri Cortex decreased ACAT activity, whereas Gardenia Fructus had no significant effect (Yotsumoto *et al.*, 1997). However, oral administration of orengedokuto was not able to significantly reduce fever caused by a bacterial pyrogen *in vivo* (Itami *et al.*, 1992).

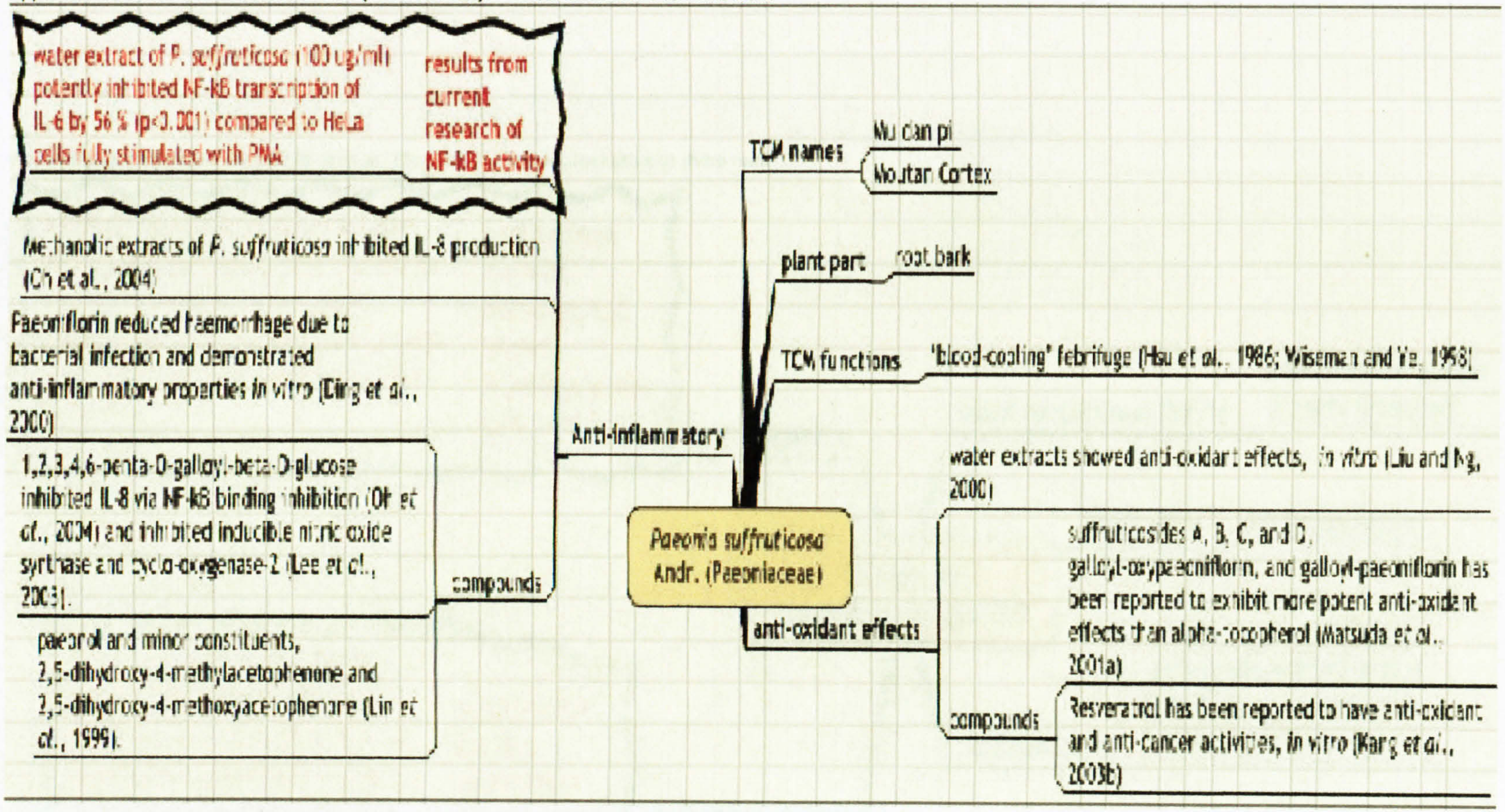
Diao-orengedokuto

Diao-orengedokuto is orengedokuto with an additional herb, Rhei Radix et Rhizoma and is traditionally used in the treatment of atherosclerosis (Kim *et al.*, 2002c). Water extract of Rhei Radix et Rhizoma showed potential anti-inflammatory effect in the currrent study (Table 8.1). Water and ethanol extracts of diao-orengedokuto has been reported to inhibit the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase more potently than extracts of orengedokuto (Kim *et al.*, 2002c). Of the herbal constituents of dia-orengedokuto, Coptidis Rhizoma was more potent at reducing HMG-CoA reductase activity, followed by Rhei Rhizoma (Kim *et al.*, 2002c). Constituents of bear bile (chenodeoxycholic acid and cholic acid) have also been reported to inhibit HMG-CoA reductase, which leads to reduction in hepatic cholesterol levels (Björkhem *et al.*, 1993).

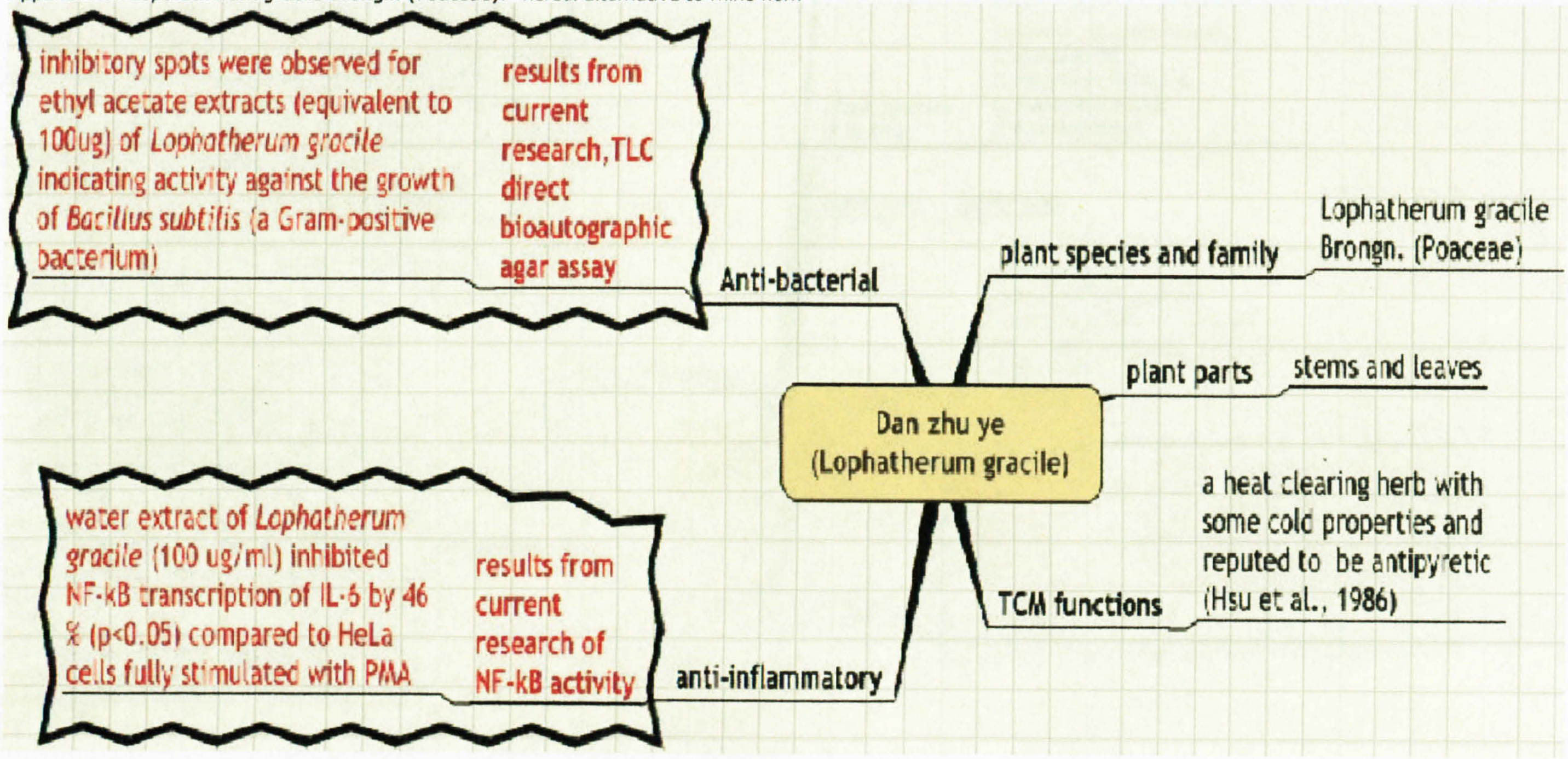
Appendix Xa. Ban lan gen, root of *Baphicacanthus cusia* Bremek (Acanthaceae):- herbal alternative to rhino horn



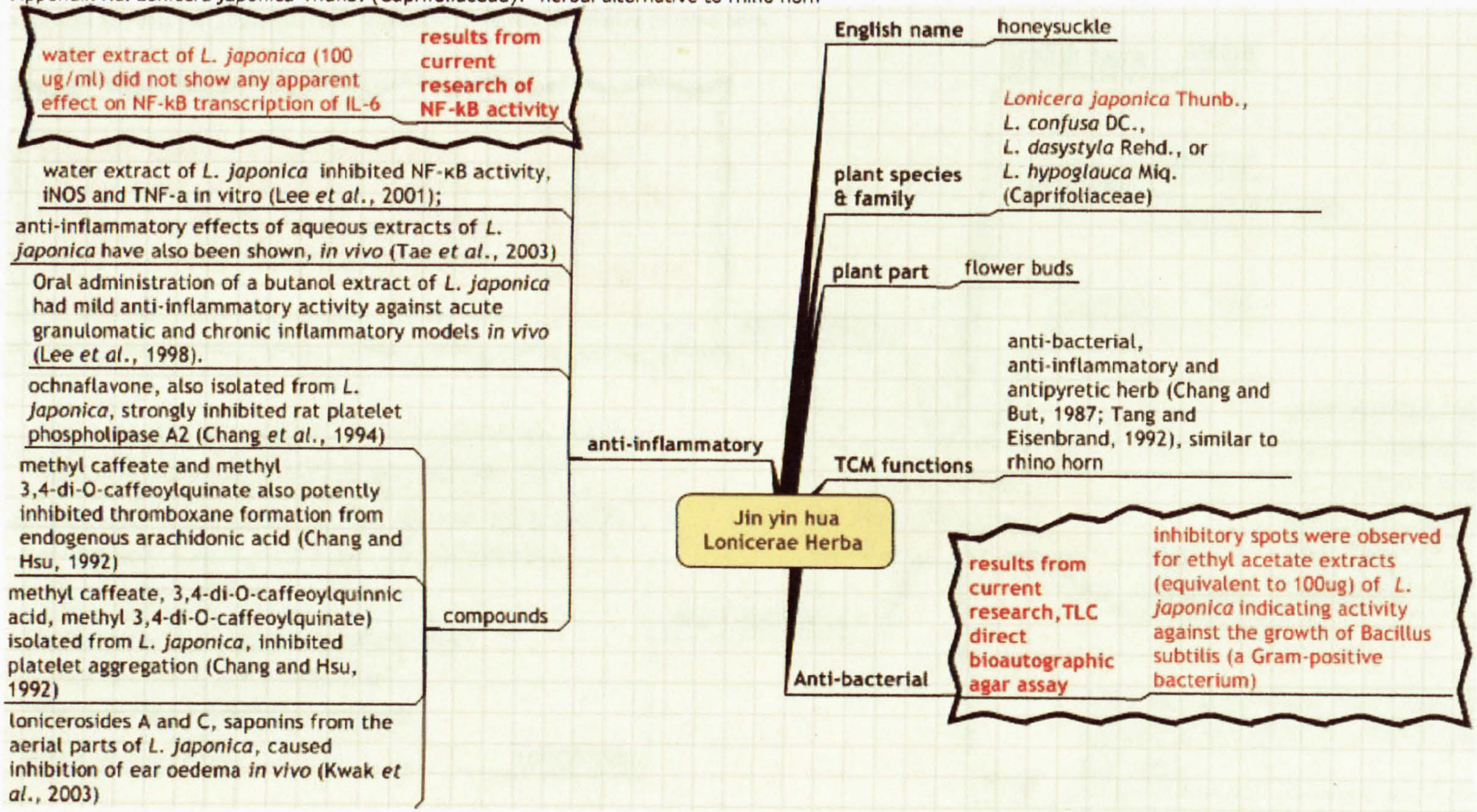
Appendix Xb. *Paeonia suffruticosa* Andr. (Paeoniaceae):- herbal alternative to rhino horn



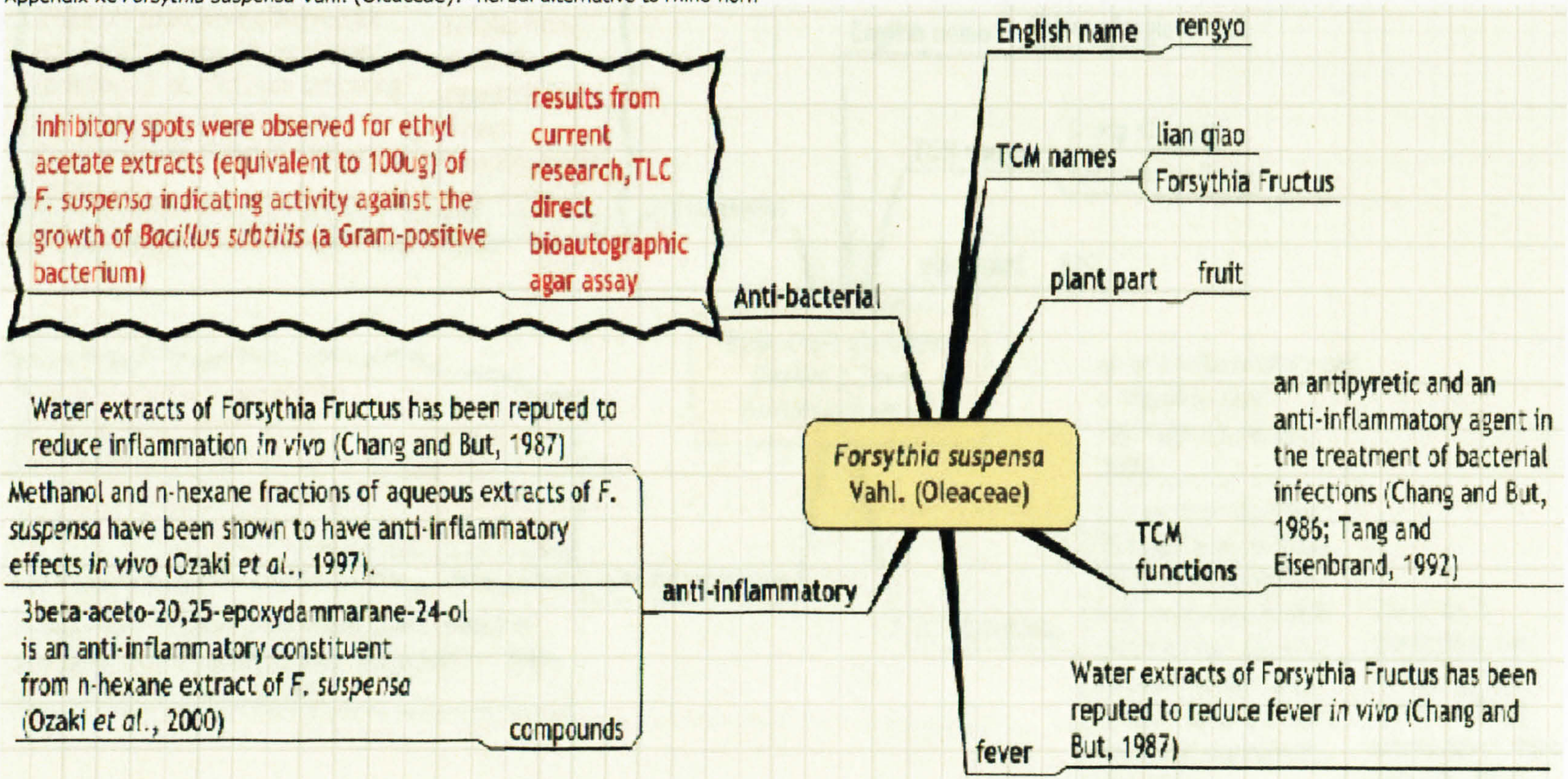
Appendix Xc. *Lopthatherum gracile* Brongn. (Poaceae):- herbal alternative to rhino horn



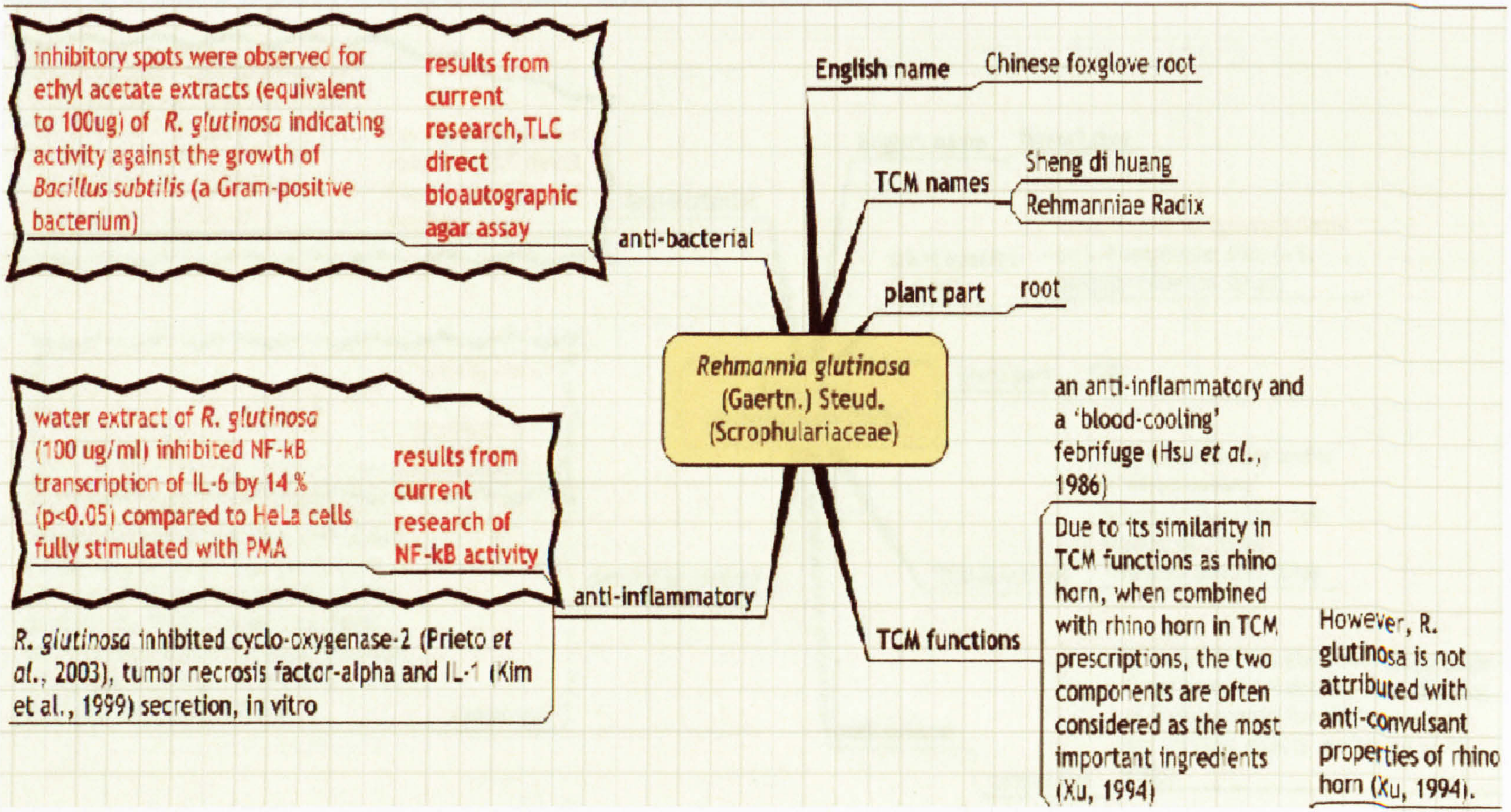
Appendix Xd. *Lonicera japonica* Thunb. (Caprifoliaceae):- herbal alternative to rhino horn



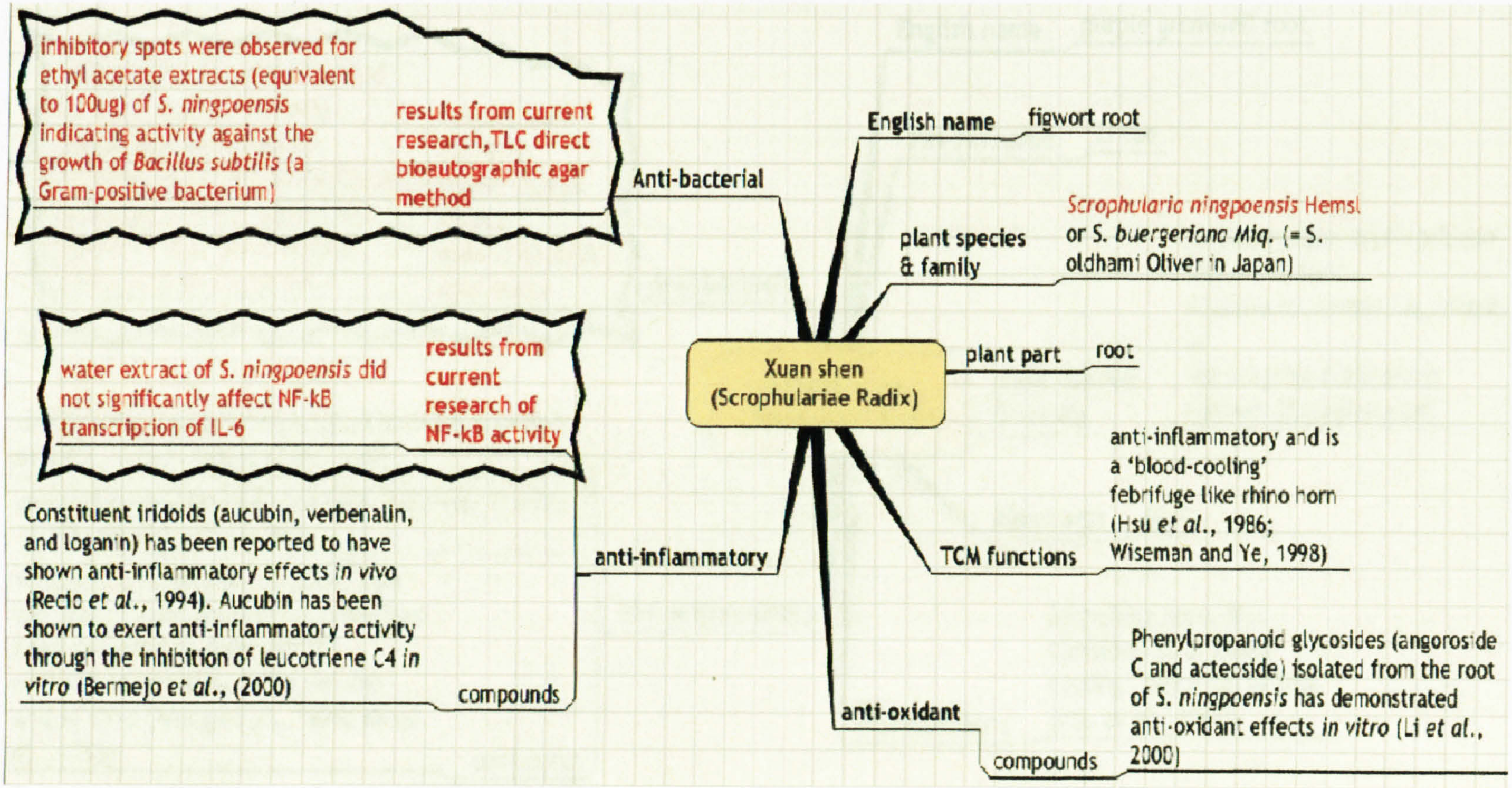
Appendix Xe *Forsythia suspensa* Vahl. (Oleaceae):- herbal alternative to rhino horn



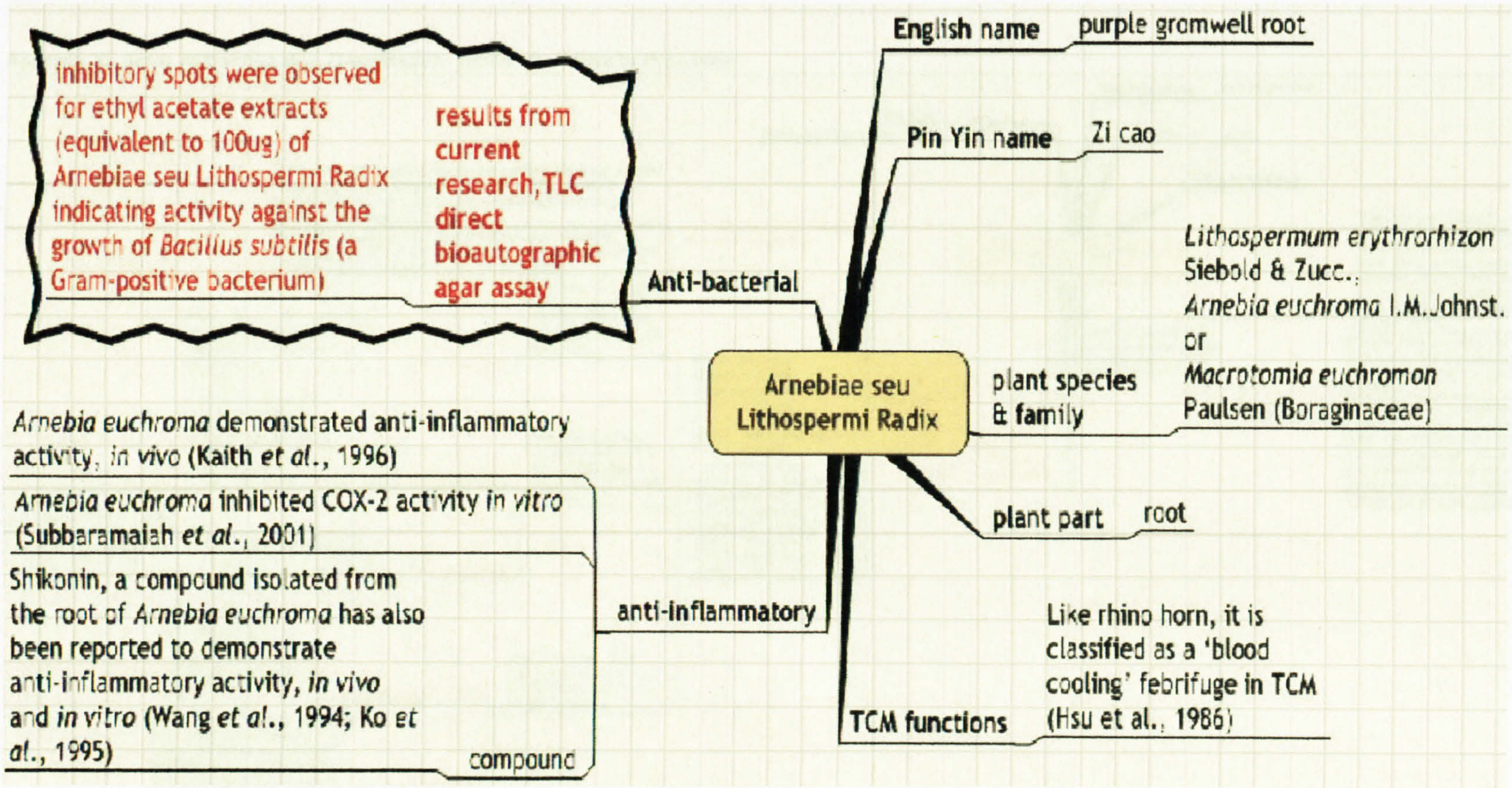
Appendix Xf. *Rehmannia glutinosa* (Gaertn.) Steud. (Scrophulariaceae):- herbal alternative to rhino horn



Appendix Xg. *Scrophularia ningpoensis* Hemsl (Scrophulariaceae):- herbal alternative to rhino horn



Appendix Xh. Arnebiae seu Lithospermi Radix (Boraginaceae):- herbal alternative to rhino horn



Appendix XI. *Salvia miltiorrhiza* Bge. (Lamiaceae):- herbal alternative to rhino horn